University of Alberta

Isolation and Characterization of *Caenorhabditis briggsae* Sex Determination Mutants

by

Carlos Egydio de Carvalho ©

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Abstract

C. elegans and *C. briggsae* are two phylogenetically related species, successfully occupying the same ecological niche and facing similar environment stresses. Their body types, developmental programs and behavior are under identical selection forces that somehow have kept their co-existence ecologically sustainable. However, similarities at the morphological level are not reflected in the genome. Indeed, both species have an incredible level of genetic divergence. An example of that is seen in the genes that make part of the sex determination pathways (SDPs) in these two species. The nematode SDPs harbor some of the most divergent genes among *Caenorhabditis* species. Here I report the isolation of *C. briggsae* sex determination mutants in an attempt to test whether the observed sequence divergence between orthologues is underlying functional changes at the protein level. The isolation and genetic analysis of *C. briggsae tra-2, tra-3, tra-1,* and *tra-2* suppressors such as *fem-2,* suggest that while functional conservation is present in the somatic SDP, the mechanism that regulates sexual fate in the hermaphrodite germline differs considerably between *C. elegans* and *C. briggsae*. In particular, *fem-2*, a gene necessary for hermaphrodite spermatogenesis in *C. elegans*, does not play the exact same role in *C. briggsae*. In addition, other *C. briggsae tra-2* suppressors do not control the onset of spermatogenesis in hermaphrodites as they do in *C. elegans* though they are needed for somatic feminization in these animals. I concluded that the regulation of germline fate that ultimately produces similar hermaphroditic outcomes is accomplished through different molecular mechanisms in these two species. For instance, the SDP in *Caenorhabditis* may be particularly tolerant to genetic changes that modulate the fate of the germline.

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List of Abbreviations

Amp	ampicilin
ARF	ADP-ribosylation factor
b	base
bp	base pair
BSA	Bovine serum albumin
C	chromosome
Cam	chloramphenicol
Cb	*Caenorhabditis briggsae*
cbp	CREB-binding protein
Cdk	cyclin-dependent protein kinase
Ce	*Caenorhabditis elegans*
CECT	FEM-2 C-terminal domain
CPEB	cytoplasmic polyadenylation element binding
Cr	*Caenorhabditis remanei*
DAZ	deleted in azoospermia
DIC	differential interference contrast (Nomarski)
DM	DSX and MAB-3
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DRE	direct repeat element
DSD	developmental system drift
DTC	distal tip cell
EDTA	ethylenediamine tetra-acetic acid
ENU	N-nitroso-N-ethylurea
EMS	ethyl methanosulfate
fbf	*fem-3* binding factor
GL	germline
gf	gain-of-function allele
GFP	green fluorescent protein
GPC	somatic gonadal precursor cells
GST	Glutathione S-transferase
HYCB	*C. briggsae* FEM-2 hydrophilic domain
HYCE	*C. elegans* FEM-2 hydrophilic domain
HYCR	*C. remanei* FEM-2 hydrophilic domain
ic	intracellular domain of TRA-2
IgG	immunoglobulin
IL	interleukin
IPTG	isopropyl-1-thio-beta-D-galactopyranoside
kb	kilobase
kbp	kilobase pair
kDa	kilodalton
lf	loss-of-function allele
LMB	leptomycin

m	milli
M	molar
m+, m-	maternally expressed mRNA
MAPK	mitogen-activated protein kinase
mdm	murine double minute
mel	maternal effect lethal
mep	MOG-interacting factor
mx	mix phenotype alleles of tra-2
NGM	nematode growth media
NXF	nuclear export factor
ORF	open reading frame
PAX	paired box
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline, Tween
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PP2C	protein phosphatase type 2C
PPDA	FEM-2 phosphatase domain
Rb	Retnoblastoma protein
REF(APE)	apurinic/apyrimidinic endonuclease
rme	receptor-mediated endocytosis
RNAi	RNA-mediated interference
SD	sex determination
SDS	sodium dodecyl sulfate
SPE	sperm
TE	Tris-EDTA
TF	transcription factor
TBS-T	Tris-buffered saline
TSP	temperature sensitive period
UTR	untranslated region
z+,z-	zygotically expressed mRNA

1.0 Introduction

1.1 Genomic era

In the last 15 years, the genomes of over 100 organisms, including human, have been completely sequenced. The amount of information arising from these projects is far greater than the capacity of geneticists to analyze and interpret them. Yet it is clear, even now, the enormous impact that sequenced genomes will have in all areas of biology for years to come. One research front to profit from this analysis is the growing field of developmental regulatory pathway evolution.

A tendency exists among biologists to use the genomic data to make evolutionary assumptions about the functional significance of the presence or absence of orthologues in different species. The immediate correlation of an identifiable homologue with a function or the prediction of no function in a divergent homologue, are often misinterpretations of the evolutionary forces involved. In fact, genetic analysis that goes beyond the identification and expression of certain genes in a given context is essential to judge the relevance of homology at the DNA level. This next level of genetic and functional organization is the pathway. The modular nature of biological networks, which can be separated in fairly independent functional units, supports the notion that the genetic pathway is a relevant level of organization upon which evolution works (Huynen et al., 2005).

The accumulating genomic data combined with thorough characterization of genetic pathways in different model organisms have increased our understanding of complex genetic networks. It is clear today that approaches focused on how evolutionary forces are "filtered" by greater levels of genetic organization are necessary to address complex trends in the evolution of form and function.

1.2 Conserved and divergent genetic pathways

Maybe the most astounding discovery in this new genomic era has been the observation that from a developmental standpoint, all animals are fundamentally similar. The conservation of the basic developmental architecture across taxa is especially hard to interpret if one wants to explain life's numerous forms by reading the information in the genes. Thus, genetic pathways that control different levels of cellular homeostasis, differentiation and senescence are overall conserved. Examples of that are seen in the conservation of signaling transduction pathways mediated by Mitogen-Activated Protein Kinases (MAPK) from yeast to humans (Widmann et al., 1999), and the role of Hedgehog and Wingless/Wnt signaling in embryo development and morphogenesis (Schneider et al., 1999; Dierick and Bejsovec 1999).

Studies of molecular embryology have pointed to an alternative explanation for the conundrum of the diversity in body plans in organisms otherwise wired by conserved genetic pathways. By selecting for specific usages of the same genetic toolkits, different developmental programs can arise without the necessity of specifically re-creating entire programs (True and Carroll 2002). That is to say that more important than the functional identity of an individual protein, or even a pathway, is the temporal control of its expression/usage and the precise combination of its activity with other pathways in the same cells during development. In the same way that one can create different robots with the same combination of Lego pieces, evolution could act to select novelties in the interactions and organization of genetic pathways that have been conserved due to their individual essential roles. In this way, modification of specific developmental programs could occur without extensive genetic change. For instance, take the example of brain development. Neuroanatomical and embryological data pointing to the differences between the organization and ontogeny of the mouse and fly brains suggest a distinct evolutionary origin for these organs. Recently, the analysis of the molecular mechanisms underlying the development of brain in these animals has suggested a very different story. In mouse and *Drosophila*, the correct expression of Hox genes in an antero-posterior pattern is necessary for specification of segmental neuronal identity in the posterior brain (Graham et al., 1989). In addition, the development of the rostral brain in both animals depends on the expression of Cephalic Head Gap genes (otd/Otx) (Hirth et al., 1995; Simeone et al., 1992; Matsuo et al., 1995). Finally, the role of paired-box (Pax) genes in forebrain development, most notably the eye structures, is also required in *Drosophila* and mouse (Kammermeier and Reichert 2001; Callaerts et al., 1997). Furthermore, some of these genes are able to functionally substitute for each other even though the most recent ancestor for these species lived over 600 million years ago (Acampora et al., 1998; Leuzinger et al., 1998; Awgulewitsch and Jacobs 1992). Therefore, conserved roles for homologous regulatory genes in central events of brain development such as patterning and regionalization of neurons are the building blocks of two anatomically very different organs. It has been proposed that functional conservation of genetic pathways involved in development may indeed be the norm in evolution (Kammermeier and Reichert 2001). Evolution may not be always explained by comparing gene sequences, but its working can usually be seen on higher levels of genetic organization.

However, not all genetic pathways are conserved. Important biological systems whose genes show rapid divergence of sequence and function across taxa are more rare, but do exist. In that respect, the immune system is a classic example of diversity. Rapid diverging cytokines and their correspondent receptors as well as the introduction and elimination of crucial molecules are observed between mammals and fish. The mammalian class II helical cytokines that include lambda interferon and IL-10 are completely absent in fish, indicating that the maturation of the mammalian and fish immune systems relies on different molecules. Similar variations can be explained by the expanding nature of these gene families that have an evolutionary history characterized by domain shuffling, deletion and gene duplication (Lutfalla et al., 2003). Rapid evolution is also apparent in more general cellular mechanisms of species in the same taxa. Certain aspects of the circuit that controls cellular senescence have diverged to a degree unimaginable for such a critical molecular control. The role of the cyclin-dependent kinase p16 in activating pRb and the role of ARF in negatively regulating p53 through MDM2 are apparently redundant regulatory circuits that respond to stress by establishing senescence in vertebrates (Collins and Sedivy 2003). Surprisingly, the result of activation of these two circuits is different between mouse, chicken, fugu and human. Such differences may explain the variability in controlling certain aspects of the pathway. For instance, loss of p53 immortalizes mouse but not human cells. Similarly, human cells can be immortalized by telomerase expression whereas mouse cells cannot (Collins and Sedivy 2003). Finally the oncogene Ras induces ARF activity in the mouse but not human cells (Collins and Sedivy 2003).

No other biological system is as diverse as those involved in determining the sexual fate of multicellular animals (for review see Haag and Doty 2005). Sex-specific differences, in the form of mating types, can be seen in organisms as primitive as yeast, though morphological changes between sexes are characteristic of higher eukaryotes. In most multicellular organisms that reproduce sexually, the common norm is the presence of two distinct sexes (dimorphism). Sexual dimorphism is an ancestral trait, shared by vertebrates and invertebrates and tracing back to the bilaterian common ancestor. Given the fact that sex is the very mechanism underlying the species success in an evolutionary scale, a reasonable expectation is that animals should share a conserved molecular pathway directing sexual development. Instead, the mechanisms of sex determination in the animal models studied so far are mostly unrelated. The lack of apparent homology between genes, differences in the initial signal that triggers the molecular cascade (e.g. environmental cues like temperature and population density or chromosome-based signals like heterogametic chromosomes or ratio of sex chromosomes to autosomes) and the very nature of the control (e.g. transcription regulation, RNA-splicing) complicate any attempt to re-construct the evolutionary history of sex determination pathways at the level of phyla. A rare exception is the role of the DM-transcription factor family of genes that includes the *Drosophila doublesex*, *C. elegans mab-3* and human *dmrt-1* as final regulators of male fate. These factors stand alone in linking different sex determination systems to a common ancestry (Raymond et al., 1998). Finding signs of homology in systems specified by drastically different genetic mechanisms is the biggest challenge in understanding the evolution of sex determination pathways.

Given the diversity of sex determination systems, separating homology from convergent evolution becomes a serious problem when the models studied to this date are few and phylogenetically far apart. Thus, the study of the evolution of molecular pathways depends on: i) a detailed characterization of the pathway at the genetic and molecular levels and ii) the availability of orthologues in sister species acting in an equivalent pathway. By comparing the conservation of gene sequences between orthologues with their respective functions in the pathway, single evolutionary steps can be identified. Furthermore, only by looking at closely related species can the meaning of diverging homologous characters be understood (True and Haag 2001).

While other rapid-evolving molecular pathways exist in a range of different animal models, very few of them have been studied in closely related species. I have started to characterize the genes of the sex determination pathway in *Caenorhabditis briggsae*, a sister species of *C. elegans*, in an attempt to collect information that will ultimately help understand how the sequence/functional changes of homologous genes are affected by their topology (e.g. specific interactions with upstream and downstream genes) on the sex determination pathways of *Caenorhabditis* species.

1.3 Evolution and pathway constraints

Do molecular pathways primarily buffer or boost evolutionary change? How does the structure of a pathway influence evolution? Are genes at different positions along a pathway prone to different evolutionary pressures? These are reasonable questions to ask if one wishes to evaluate the evolutionary history of a gene that plays a given role in a molecular pathway of any organism.

I asked two more practical questions in view of the system I decided to work with. How are homologues involved in sex determination changing in closely related species, and what are the consequences of these changes in the evolution of the pathway as a whole? If sexual dimorphism and the underlying developmental programs are the same in both nematode species (see below), then the changes in the sequences of sex-determining proteins observed between them have not altered the ultimate phenotypic outcome mediated by the pathway. This being true, it follows that there must be a level of genetic flexibility that allows, even in rapid evolving pathways, the preservation of the original phenotypic outcome while exploring different molecular "ways" to do so. That is not to say that individual genes will not acquire or lose specific functions in the pathway, but that these changes are constrained by the function of the pathway as a whole. Presumably, diverging pathways that while evolving still managed to maintain the ancestral developmental role in the organisms accomplished that through co-evolution of their protein members. Consequently, pathways underlying homologous characters are not necessarily static, instead these systems can undergo rapid evolution as long as they diverge inside the functional boundaries defined by their evolutionary history. The process behind the divergence noticed in such morphogenetic or regulatory blueprints that accommodates the effects of genetic variation was named *Developmental System Drift* (DSD) by True and Haag (2001). In short, DSD predicts the appearance of superficially similar molecular mechanisms in different species that are not functionally interchangeable.

A good example of how DSD affects the topology of pathways is seen in the pathway for vulval development in *Caenorhabditis* and *Pristionchus* nematodes. In both, *lin-39* is involved in vulval development as seen by the Vulvaless phenotype of mutants. However, the mechanism by which LIN-39 promotes formation of the vulva is not the same. In *C. elegans*, LIN-39 is necessary to prevent vulva cells from fusing to the epidermis whereas in *P. pacificus* LIN-39 prevents apoptosis of cells destined to form the vulva (Eizinger and Sommer 1997). In fact, though the pathway underlying vulval development is homologous between these species, the specific function of a major regulator (LIN-39) has dramatically changed.

As expected, some of the best examples of DSD come from sex determination pathways. In dipteran flies, the ratio of sex chromosomes to autosomes (see below) determines a series of sex-specific splicing events that result in the downstream regulation of *doublesex (figure 1)*. In *Drosophila*, a XX genotype signals for transcription of a female-specific *sex-lethal* (*Sxl*) mRNA. SXL is a RNA-splicing factor that splices a second wave of *Sxl* transcripts later produced in both sexes through activation of a different promoter. By allowing correct splicing only in females, this regulation effectively deprives males of SXL activity. The result is that only in female flies, a second SXL target, the transformer (*tra*) mRNA, is correctly spliced. TRA (which is unrelated to *Caenorhabditis tra* genes, see below) is another female-specific splicing factor that splices the mRNA of the downstream transcription factor *doublesex*, ultimately allowing female development (Cline and Mayer 1996) (*figure 1*). Surprisingly, the *Sxl* orthologue in *Ceratitis capitata*, the Mediterranean fruit fly, does not undergo sex-specific splicing and apparently is not involved at all in sex determination in this species (Saccone et al., 1998). A similar situation is seen in *Musca domestica* (housefly) (Meise et al., 1998). In fact, though the *tra* and *doublesex* orthologues are apparently important for sex determination in all dipterans examined so far, the important role of *Sxl* in the pathway has been restricted to the *Drosophila* lineage. Since sex-specific splicing events are the underlying regulatory mechanism in the sex-determining pathways of all dipterans studied so far, how do *C. capitata* and *M. domestica* control TRA activity? The housefly has a different factor, named "F", that is analogous to, but not functionally interchangeable with, SXL (Meise et al., 1998). On the other hand, the *C. capitata* TRA is able to specifically regulate its own mRNA much in the same way the *Drosophila* SXL. As in *Drosophila*, a female-specific *tra* product is produced allowing correct activation of *doublesex* only in XX flies (Pane et al., 2002).

Therefore, significant divergence can occur in pathways that are otherwise functionally conserved. I decided to investigate to which extent the sequence differences noticed between *C. elegans* and *C. briggsae* sex determination orthologues are reflected into functional changes (see below) and how the sex determination pathway in *C. briggsae* has coped with the pressures imposed by genetic variation and functional conservation.

1.4 *Caenorhabditis sp.*

Of the 17 species of *Caenorhabditis* described to date, *C. elegans*, *C. briggsae* and *C. remanei* are the most frequently used for research in the lab. These three species are morphologically very similar, their major difference being the strategy of reproduction. *C. elegans* and *C. briggsae* are hermaphrodite/male species while *C. remanei* is a male/female species.

C. briggsae shares the same characteristics that make *C. elegans* amenable to genetic and molecular analysis. Besides being small with a short and prolific reproductive cycle (~300 eggs laid in around 3 days), these soil dwelling nematodes are transparent, which allows visualization of internal organs (including the gonads) in intact, living animals. Worms are either males or self-fertilizing hermaphrodites. Hermaphrodites are somatically female worms that transiently produce a limited number of sperm that are used for self-fertilization in the adult life. The progeny derived from a self-fertilized hermaphrodite is made up of genetically similar worms. The inbreeding associated with hermaphrodites such as *C. briggsae* and *C. elegans* facilitates the establishment of homozygous strains and the control of the flow of alleles across generations, a feature particularly attractive to geneticists. Males, on the other hand, become "vectors" with which to introduce new alleles into the gene pool of a particular strain. Conveniently, after being inseminated by a male, a hermaphrodite preferentially uses the stored male sperm instead of its own, facilitating the identification of cross-progeny.

1.5 Alike, but not the same

Indistinguishable to the untrained eye, *C. elegans* and *C. briggsae* occupy the same ecological niche, have the same genome size and the same chromosome number (Nigon and Dougherty 1949; Jovelin et al., 2003). Only 800 of the 19,500 predicted *C. briggsae* genes lack *C. elegans* counterparts (Stein et al., 2003). Their earliest common ancestor is estimated to have lived 80 to 100 million years ago, around the same time that the mouse and human lines split in the mammalian evolutionary tree (Coughlan and Wolfe 2002). Contrary to what may be expected, the morphological similarity between these worm species is not reflected in the genome. Despite their almost identical appearance and similar developmental programs (Kirouac and Sternberg 2003), *C. elegans* and *C. briggsae* are genetically very different (Fitch et al., 1995; Kiontke et al., 2004). 18 rDNA and other individual gene comparisons show that genomic differences between these two species are as extensive as the differences observed between mammalian orders (Rzhetsky and Nei 1992). In fact, high rates of genetic divergence are a common feature among *Caenorhabditis* nematodes. For example, the divergence observed in the RNA polymerase-2 locus between *C. briggsae* and *C. japonica* is equivalent to the divergence observed between human and zebrafish (Kiontke et al., 2004). It has been proposed that the genetic differences in sex-determining genes could be responsible for the reproductive isolation between *Caenorhabditis* species (Baird 2002; Cutter and Ward 2005).

If a comparable amount of genetic change has taken place in the genome of mammals and *Caenorhabditis* during similar evolutionary time, how can we account for the dramatic body differences between a mouse and a human in contrast to the morphological conservation observed in *C. elegans* and *C. briggsae*?

1.6 Genome evolution and evolution of morphology

A simple explanation for this paradox is that the evolution of genomes is not necessarily reflected in the evolution of structure (King and Wilson 1975), and therefore it is possible that long periods of morphological stasis are accompanied by extensive genomic divergence (Fitch and Thomas 1997). Fast evolving genes could show considerable sequence divergence and yet play the same role. In fact, *C. briggsae* genomic variation could be constrained by natural selection in such a way as to lead to co-evolution of genes in the same pathways (see above). An alternate possibility is that positive selection exists to alter the structure of the pathway by adding or eliminating the function of its genes, ultimately modifying the original molecular mechanism. Positive selection is a driving force in the evolution of a series of highly diverse systems, most notably in the divergence of protease inhibitors (Creighton and Darby 1989) and egg-sperm recognition mechanisms (Jansa et al., 2003). An important pre-condition for positive selection is the relaxation of functional constraints. For example, α-crystallin, the major structural component of the vertebrate eye lens, is conserved among mammals but shows a high divergence rate in the blind mole rat (Wu and Li 1985). Thus, loss of selection pressure usually precedes the adoption of new functions driven by positive selection.

The sex determination pathway in *C. elegans* is ideal for this type of study for three basic reasons:

a) The genes involved, their mutant phenotypes and genetic interactions have been well established.

b) Sex-determining orthologues are evolving at a faster rate than *Caenorhabditis* orthologues involved in other molecular pathways (Hansen and Pilgrim, 1998).

c) The binary fate decision and the organization of the sex-determining pathway in negative regulatory steps facilitate genetic analysis and screening for mutants.

1.7 Sex dimorphism in *Caenorhabditis*

Virtually all tissues in the worm show some level of sexual dimorphism, making the identification of hermaphrodites and males fairly easy. The first event that creates sexual difference occurs at the end of the male embryogenesis with the apoptosis of two hermaphrodite-specific neurons (Trent et al., 1983; Conradt and Horvitz 1998). The major anatomical and behavioral differences, however, become evident later in the passage from L4 larval stage into adulthood. Hermaphrodites are characterized by the presence of a two-armed gonad that converges at the vulva, the presence of spermatheca and an undifferentiated whip-like tail (*figure 2*). The tail, which specifically in male development undergoes a series of striking morphological changes, is a hallmark of sexual dimorphism in these worms (*figure 3*). The hermaphrodite germ cells mature progressively from the distal end of the gonad towards the vulva (*figure 4*). Mature oocytes are fertilized as they pass through the spermatheca and reach the uterus. Embryos begin development within the hermaphrodite but are soon laid and allowed to complete development in the external environment. Conversely, males have a single-armed gonad where sperm is produced throughout the adult life (*figure 3*). The gonad connects posteriorly to a specialized fan-like tail harboring sensory and copulatory structures (*figure 3*). Sex-specific behaviors such as egg laying (hermaphrodites) and mating (males) are underlined by further dimorphic differences in musculature, neuronal wiring and yolk production (Kimble and Sharrock 1983; Yi et al., 2000; Shen and Hodgkin 1998). Abnormal sexual fates are therefore easily discriminated by visually identifying worms that show a combination of intersex or incompletely formed structures.

1.8 Common sex determination mutant phenotypes in the worm

Sex-determining mutants can be classed into two major phenotypic groups based on their effect on development of the soma and germline: masculinized hermaphrodites (transformer or "*tra*" phenotypes) and feminized ("*fem*" phenotypes) males (soma/germline) or hermaphrodites (germline). Loss-of-function (*lf*) mutations in sex-determining genes that cause Tra phenotypes are indicative of the feminizing nature of the wild type gene product. Conversely, Fem phenotypes are often the result of *lf* mutations of masculinizing genes. Gain-of-function (*gf*) mutations have also been important to the characterization of specific regulatory steps in the *C. elegans* sex determination pathway, in particular during translational inhibition of germline mRNAs (Zhang et al., 1997; Puoti and Kimble 2000).

All male somatic structures, mating behavior and spermatogenesis are normal in completely transformed XX Tra worms. For example, null alleles of *tra-1* in *C. elegans* cause XX worms to fully adopt the male developmental program. The vulva is not formed, male-specific neurons develop, the germline shifts toward sperm production and the tail copulatory structures are functional, allowing the worm to initiate mating behavior and successfully inseminate hermaphrodites (Hodgkin and Brenner 1977). In these strains, the hermaphrodite genotypic sex is overridden by the masculinizing allele. Because the hermaphrodite developmental program is blocked, these strains have to be maintained balanced as heterozygotes (or as homozygotes at permissive temperatures in the case of temperature sensitive (*ts*) alleles). Alleles that cause incomplete hermaphrodite-to-male transformation (intersex phenotype) are easier to detect than complete reversals due to their peculiar phenotypes when compared to both wild-type sexes. Intersex *tra* hermaphrodites fail to fully develop all or some of the somatic male structures. The degree of sexual transformation that results is variable and depends on the allele (Hodgkin and Brenner 1977). For example, adult *C. elegans* hermaphrodites homozygous for *tra-2* null alleles have a non-functional (blunt) pseudo-male tail and often retain the hermaphrodite bi-reflexed pattern of the somatic gonad. The vulva is frequently enveloped by overgrown tissue that blocks egg laying and mating (protruded vulva). Sperm production is sometimes detected in the gonad, but the lack of oocytes, copulatory structures and mating behavior prevent self or cross-fertilization (Hodgkin and Brenner 1977). Thus, homozygous strains with non-*ts* alleles resulting in incomplete masculinization of XX worms are commonly unviable due to hermaphrodite sterility and therefore need to be balanced. Male worms are unaffected by these alleles (since both soma and germline are already fully masculinized) and can be used for mating experiments.

Feminizing mutations cause the X0 soma and germline to adopt the female fate. Males can be completely transformed into females much like null *tra-1* alleles can completely transform XX animals into males (Kimble et al., 1984). For example, *C. elegans* X0 worms homozygous for a *fem-2* null allele derived from *fem-2* mothers are feminized and virtually indistinguishable from wild type hermaphrodites (Hodgkins 1986). X0 worms can also be partially feminized and develop a protruding vulva and a pseudo-male tail. In *fem* X0 worms, the germline is driven to produce large oocyte-like cells ("ooids") that become trapped inside the somatically male gonad. As with partial masculinization of XX worms, partially feminized X0 animals are sterile pseudo-females. Indeed, incomplete transformation of XX animals into males and X0 animals into females will often yield similar intersex phenotypes because of their intermediate sexual fates (*figure 5*). In XX worms, *fem* alleles have no effect on the soma, which is itself already feminized, but instead cause feminization of the germline (Hodgkin 1986; Kimble et al., 1984). Since the XX germline needs first to adopt a male identity to generate sperm cells during L4 stage, early feminization results in ablation or reduction of hermaphrodite spermatogenesis. After the switch to oogenesis, XX feminized worms show a reduced brood size or are entirely unable to self-fertilize due to reduction or complete lack of sperm cells, respectively. In *C. elegans*, *fem-2 ts* alleles cause XX sterility when worms are grown at a restrictive temperature (Kimble et al., 1984). *fem-2* worms are easily distinguished by the absence of eggs in the uterus and the presence of mature, unfertilized oocytes that accumulate in the proximal gonad region. This "stacked oocytes" phenotype is characteristic of mutations that prevent sperm production while not affecting oogenesis (*figure 6A*). In addition to the *fem* alleles, *fog* (feminization of the germline) mutations also result in "stacked oocytes" hermaphrodites (see below). Thus, different from *tra* alleles, the effects of *fem* mutations on *C. elegans* spermatogenesis can affect both X0 and XX worms, a feature that further complicates the maintenance of homozygous *fem* strains.

1.9 C. *elegans* sex determination pathway – Soma

1.9.1 Primary signal and final regulator

In *Caenorhabditis*, the ratio of X chromosomes relative to autosomal chromosomes (X:A ratio) is the initial signal that specifies the sexual fate of somatic and germline tissues. An X:A ratio of 1 leads to hermaphrodite (XX) development whereas an X:A ratio of 0.5 results in a male (X0) fate (Akerib and Meyer 1994; Nicoll et al., 1997). Worms are extremely sensitive to slight alterations of this ratio, as in seen in the case of polyploidy (Madl and Herman 1979).

The sex determination pathway works through a series of negative regulatory steps triggered by the readout of the X:A ratio that ultimately controls the activation or inhibition of TRA-1 activity (*figure 7*). TRA-1, the final master regulator of sexual fate, is a zinc-finger transcription factor responsible for up-regulating genes necessary for the female fate while repressing male development (Zarkower and Hodgkin 1992). In the absence of TRA-1 activity, the worm adopts the male developmental program as a "default", independently of its X:A ratio. Only a few TRA-1 target genes have been identified, including *egl-1* and *mab-3*. In hermaphrodites, expression of *egl-1* and *mab-3* are repressed or reduced by TRA-1, respectively preventing hermaphrodite-specific neurons from undergoing apoptosis and allowing production of yolk protein by intestinal cells, two morphological events needed for female development (Conradt and Horvitz 1998,1999; del Peso et al., 2000; Shen and Hodgkin 1998; Yi and Zarkower 2000). Another gene controlled by *tra-1* is *fhk-6*. FHK-6 is a forkhead transcription factor involved in gonad development in both sexes. Specifically in males, however, *fhk-6* acts in the early establishment of sexual dimorphism in primordial gonadal cells. Males mutant for *fhk-6* attempt to form a hermaphrodite gonad but never succeed. Though necessary for somatic differentiation, *fhk-6* does not affect the germline fate (Chang et al., 2004). Thus, so far *fkh-6* and *mab-3* are the only known soma-specific sex determination genes downstream of *tra-1*.

In the past two decades, genetic screens have yielded a number of *C. elegans* mutants that directly or indirectly affect sex determination. Today, over 15 genes involved in activating or repressing TRA-1 in the germline and/or the soma have been isolated and characterized (*table 1*) (for review see Goodwin and Ellis 2002). Due to the regulatory character of the pathway, genetic analysis of double mutants has allowed the researchers to arrange the order in the pathway based on the epistatic relationships between sex-determining genes. Although the precise biochemical signaling mechanism that carries the X:A information downstream to activate/repress TRA-1 remains for the most part unclear, a precise genetic relationships between these genes have been known for some time (*figure 7*).

1.9.2 Translating the X:A ratio

What elements of the X:A ratio are perceived by the worm? Though no clear autosomal element is known, two X-chromosome genes, *sex-1* and *fox-1*, have been associated with the ability of the worm to correctly interpret its genotypic sex. When over-expressed, *sex-1* and *fox-1* have feminizing activity in X0 males while reducing their expression causes masculinization in XX worms, presumably by altering the ultimate X:A value. SEX-1, a member of the nuclear hormone receptor family (Carmi et al., 1998) and FOX-1, an RNA-binding protein (Nicoll et al., 1997; Skipper et al., 1999), switch on the correct sex determination program by negatively regulating the expression of their target gene, *xol-1*, in the XX embryo.

1.9.3 xol-1 and the sdc genes

The initial response to the X:A ratio triggers downstream events that ultimately control both sex determination and dosage compensation. The latter is a necessary sex-specific adjustment in the expression of X-linked genes to make up for the unequal dose of X chromosomes in males and hermaphrodites. In *C. elegans*, this is achieved by XX-specific down-regulation in transcription of X-linked genes (Meyer and Casson 1986; Dawes et al., 1999; Donahue et al., 1987). The genetic regulation of both processes depends on the activity of *xol-1* and the *sdc* genes. The failure to reduce X-linked transcription in hermaphrodites (XX) or the abnormal reduction in males (X0) frequently results in sex-specific lethality.

XOL-1 activity detected in X0 worms is needed to direct male development as well as to maintain high expression levels of X-linked genes (Miller et al., 1998). The absence of XOL-1 activity in hermaphrodites leads to active reduction of the expression levels of genes on both X-chromosomes. This is accomplished by expressing three genes; *sdc-1*, *sdc-2* and *sdc-3*. Because dosage compensation defects are often embryonic lethal, null mutations in *xol-1* and *sdc-2* result in X0 and XX-specific lethality, respectively (Nonet and Meyer 1991; Villeneuve and Meyer 1990; Nusbaum and Meyer 1989; Delong et al., 1993).

XOL-1 affects dosage compensation and sex determination in the male by negatively regulating the expression of *sdc-2* (Rhind et al., 1995). In hermaphrodites, SDC-2 and SDC-3 co-localize on X-chromosomes where they are thought to form a complex responsible for down-regulating X-linked transcripts (dosage compensation pathway) (Davis and Meyer 1997). Additionally, SDC-2 prevents XX masculinization by repressing the expression of another male-specific gene, *her-1*, and consequently triggering downstream steps in the sex determination pathway that lead to the female fate (Nusbaum and Meyer 1989).

1.9.4 Masculinizing genes

The steps downstream from HER-1 only govern sex determination and do not affect dosage compensation. Null mutations in *her-1* and in any of the FEM complex genes (*fem-1*, *fem-2* and *fem-3*) cause feminization of both the soma and germline of X0 animals (Doniach and Hodgkin 1984; Hodgkin 1986; Kimble et al., 1984) and in the case of the *fem* genes, also of the XX germline (impaired spermatogenesis). The abnormal sex phenotype in these mutants points to a masculinizing role for HER-1 and the FEM proteins.

Like *xol-1*, *her-1* is necessary only for male development (Hodgkin 1980). The cell non-autonomous activity of HER-1 (Hunter and Wood 1992) and the presence of a secretory signal in the protein sequence (Perry et al., 1993) suggest a role as an extra-cellular signaling molecule. It has been demonstrated that HER-1 activity depends on its binding (Hodgkin 1980) and consequent repression of the TRA-2 transmembrane receptor (Okkema and Kimble 1991). Thus, male development depends on a coordinated intercellular signal molecule responsible for synchronizing the fate decision of neighboring cells.

The FEM proteins form a major cytoplasmic regulatory complex that represses the female-promoting activity of TRA-1 in the male (Hodgkin 1987). Genetic analysis places all three *fem* genes "squeezed" between the feminizing proteins TRA-2 (upstream) and TRA-1 (downstream) (*figure 7*). The FEM proteins are the cell-autonomous carriers of the male signal transduced by HER-1. How the FEM proteins receive and convey this signal remains largely unknown. Recent data supports a role for the FEM complex in controlling the subcellular localization of TRA-1. In males, FEM activity prevents female fate by keeping cytoplasmic TRA-1 levels high. In XX worms, TRA-2 sequesters the FEM complex to the membrane and nuclear TRA-1 levels rapidly increase, presumably triggering the female transcriptional regulation program (Jager et al., 2004).

Yeast two-hybrid and co-immunoprecipitation assays have shown that all three FEM proteins interact with each other (Chi-Sang and Spence 1996; Tang et al., 2001). The current model that explains how the FEM complex transduces the HER-1 signal to ultimately repress TRA-1 is based on the biochemical nature of FEM-1 and FEM-2 proteins. FEM-1 contains ankyrin repeats, which have been associated with protein-protein interactions (Spence et al., 1990). FEM-2 is a serine/threonine phosphatase of the PP2C family (Pilgrim et al., 1995). Although its substrate in the pathway remains unknown, there is evidence suggesting that the phosphatase activity of FEM-2 is essential for male development and spermatogenesis (Hansen and Pilgrim 1998).

1.9.5 Feminizing genes

TRA-1 and the SDC proteins are not the only feminizing factors in the sex determination pathway. Loss-of-function mutations in two other genes, *tra-2* and *tra-3* also cause masculinization of hermaphrodites while having no effect in males (Hodgkin and Brenner 1977).

Genetic studies place *tra-2* and *tra-3* upstream of the *fem* genes (Hodgkin 1986). TRA-2 is a transmembrane receptor thought to bind HER-1 extracellularly (Kuwabara et al., 1992; Kuwabara 1996a) and FEM-3 through its cytoplasmic domain (Mehra et al., 1999). In the absence of HER-1, TRA-2 activity indirectly allows TRA-1-mediated transcriptional control in XX animals by binding and restricting the FEM complex to the membrane domain. The reduction of free cytoplasmic FEM complex prevents it from inhibiting TRA-1. Accordingly, over-expression of the intracellular domain of TRA-2 or the disruption of the putative HER-1 binding site feminizes X0 animals by reducing the availability of free FEM proteins in the cytoplasm (Kuwabara and Kimble 1995; Kuwabara 1996a).

The recent discovery that SEL-10, an E3 ubiquitin protein ligase, targets FEM-1 and FEM-3 to degradation (Jager et al., 2004) suggests the following biochemical model to explain the transduction of the sex determination signal: In hermaphrodites, TRA-2 activates a putative protein kinase that phosphorylates FEM-1 and/or FEM-3, targeting the complex for ubiquitination and proteosomal degradation. Reduction of cytoplasmic FEM proteins results in TRA-1 nuclear localization. In males, HER-1 prevents activation of the kinase by binding to TRA-2. Without TRA-2-mediated repression, FEM-2 can dephosphorylate FEM-1 and/or FEM-3. The dephosphorylated form of the FEM complex is then able to directly or indirectly regulate TRA-1 cytoplasmic localization (Jager et al., 2004). The role of a kinase in the pathway is predicted based on the functional importance of FEM-2-mediated dephosphorylation for correct sexual decisions (Pilgrim et al., 1995). The failure to identify the particular kinase involved in controlling the activity of the *fem* genes after innumerous genetic screens strongly suggests that its activity is ubiquitous and likely essential for survival. Another possible explanation is that the FEM complex is a substrate for more than one kinase. A redundant kinase activity in this step of the pathway would complicate the isolation of a mutant in genetic screens. However, the apparent co-evolution of rapidly evolving genes (see Discussion) in the pathway and the lack of another example of functional redundancy among sex-determining proteins argue against this explanation.

Genetic analysis of the *C. elegans tra-3* gene indicates that it shares the same position in the sex determination pathway as *tra-2*. Because the effect of TRA-3 in somatic feminization appears to be secondary, it has been assumed to act as a co-factor of TRA-2. TRA-3 is a calpain protease that cleaves and releases the intracellular domain of TRA-2 (TRA-2ic) (Sokol and Kuwabara 2000). The TRA-2ic fragment contains the FEM-3 binding domain and once free in the cytoplasm could act to amplify the inhibition of the FEM complex. The TRA-2ic peptide has an intrinsic, though weak, feminizing activity that cannot substitute for the function of the undigested TRA-2 protein in the soma. Interestingly, these fragments can bypass the downstream step in the pathway mediated by the FEM proteins to directly bind and activate TRA-1 (Lum et al., 2000). Although the role of this interaction remains to be determined, the TRA-2ic-TRA-1 complex could represent a secondary feminizing loop, branched out from the main pathway during evolution. Conversely, the TRA-2ic-TRA-1 interaction could be reminiscent of the primitive pathway layout that transduced the sex determination signal before the introduction of the *fem* genes.

Furthermore, in *C. elegans*, a smaller *tra-2* transcript encoding only the cytoplasmic domain of TRA-2 is expressed in the germline (*tra-2b*). As with the case of TRA-2ic, TRA-2b can directly bind TRA-1. The interaction of TRA-2b with TRA-1 is essential for spermatogenesis as seen by the complete germline feminization of worms with mutation in the TRA-1 binding domain of TRA-2 (*mx* alleles) (Lum et al., 2000). Thus, a soluble TRA-2 peptide with a weak feminizing activity is available in the soma (TRA-2ic) through TRA-3-mediated proteolysis of TRA-2 and in the germline (TRA-2b) through translation of a second *tra-2* transcript (Lum et al., 2000).

1.9.6 Soma vs germline sex determination

During development, somatic tissues that show sex dimorphism in the adult adopt either a female or male fate through the regulation of TRA-1-mediated transcription. The male fate achieved through TRA-1 repression is ensured by negatively regulating the activity/expression of all feminizing factors (SDC-1, SDC-2, SDC-3, TRA-2) in the sex determination cascade. In the hermaphrodite soma, the steps mediated by male-specific factors (XOL-1, HER-1, FEM-1, FEM-2, FEM-3) must be inhibited to ensure TRA-1 activity and female development.

In X0 worms, both the soma and germline choose a male fate, ensuring not only the development of necessary somatic structures but also ongoing spermatogenesis throughout adulthood. Hermaphrodites, however, first produce sperm before switching to oogenesis. The germline in XX worms must therefore be able to initially bypass the signal from the X:A ratio to enter spermatogenesis and later adopt it to allow oocyte production. The re-setting of the developmental program in the hermaphrodite germline is possible because of the existence of specific factors that transiently modulate the feminizing signal in this tissue.

1.10 Germline sex determination

In contrast to the soma, the *fem* genes are epistatic to *tra-1* and occupy the final regulatory step in the germline sex determination pathway (*figure 7*). Activation/repression of these male-determining genes is what ultimately drives the production of sperm or oocytes (Hodgkin 1987; Schedl et al., 1989).

As in males, sperm production in hermaphrodites depends on the inactivation of TRA-2. Unlike males, however, XX worms cannot rely on HER-1-mediated repression of TRA-2, since *her-1* expression is inhibited by SDC-2 activity as a consequence of the X:A ratio (Trent et al., 1991; Hodgkin 1980). Therefore, spermatogenesis in XX worms requires a unique set of germline-specific factors that transiently inhibit TRA-2 activity in the germline while not affecting the correct somatic fate decision. Remarkably, all germline-specific control of sex-determining factors occurs at the translation level. The hermaphrodite germline is initially enabled to produce sperm through translational repression of *tra-2* mRNA (Goodwin et al., 1993). RNA-binding proteins such as LAF-1 (Goodwin et al., 1997) and GLD-1 (Francis et al., 1995a,b; Jan et al., 1999) bind repeat elements (DRE) present in the 3-UTR of the *tra-2* mRNA to prevent its translation. GLD-1 activity depends on binding of another protein, FOG-2, to form a complex needed for *tra-2* repression. Though FOG-2 does not directly bind the DRE, it is essential for the formation of the GLD-1 repression complex (Clifford et al., 2000). The LAF-1 and GLD-1 repression mechanisms appear to be independent from each other, leaving the question of how the repression is coordinated. The lack of TRA-2 in XX germ cells is thought to release the downstream masculinizing genes from inhibition and promote male fate in a tissue-specific manner. The main consequence of *tra-2* inhibition is the onset of spermatogenesis in the germline. Along with *fem-1*, *fem-2* and *fem-3*; two other germline-specific genes, *fog-1* and *fog-3*, play a role in spermatogenesis (Barton and Kimble 1990; Ellis and Kimble 1995). The mechanism through which the *fem* and *fog* genes trigger the onset of spermatogenesis is completely unknown.

The *tra-2* mRNA repression machinery important during XX spermatogenesis is dispensable in males (Schedl and Kimble 1988; Francis et al., 1995a) since X0 worms use HER-1 to constitutively repress TRA-2 in the germline as well as in the soma (Kuwabara and Kimble 1992).

Another XX-specific event in germline development is the switch to oogenesis with the re-adoption of the female fate during the L4 larval period. The primary signal that triggers the sperm-oocyte switch is a change in the TRA2:FEM-3 protein ratio in the germline. Until mid L4, when sperm production takes place, there is more FEM-3 than TRA-2 activity in the germline. As adulthood approaches, TRA-2 activity becomes more prevalent (Goodwin and Ellis 2000). This is accomplished through timely repression of *fem-3* in the XX germline. Similar to *tra-2* regulation during XX spermatogenesis, changes in the TRA-2:FEM-3 ratio are achieved by post-transcriptional inhibition of the *fem-3* mRNA. Two proteins, FBF-1 and FBF-2, have been shown to bind the 3' UTR of the *fem-3* mRNA (Zhang et al., 1997). Mutations in the 3'UTR of *fem-3* prevent oogenesis and result in continuous production of sperm in the adult hermaphrodite (Barton et al., 1987; Ahringer and Kimble 1991). Additional factors involved in *fem-3* repression include NOS-3 and the *mog* genes. NOS-3 is a member of a Nanos-like protein family in *C. elegans* that includes NOS-1 and NOS-2 (Kraemer et al., 1999). NOS-3 binds to the FBF proteins to form a protein complex involved in translational repression of *fem-3* mRNA. Another mechanism for reducing FEM-3 activity in the germline depends on the activity of the MOG proteins. The *mog* genes (*mog-1* to *6*) were isolated in a screen for mutants that fail to make the switch to oogenesis (Graham et al., 1993; Graham and Kimble 1993). MOG-1, 4 and 5 contain DEAH box domains similar to the yeast RNA helicase PRP16 which is involved in RNA splicing (Puoti and Kimble 1999). It is not yet known whether the MOG proteins regulate *fem-3* by directly binding its mRNA (Gallegos et al., 1998) though recent data seem to suggest that *mog-6* encodes a divergent nuclear cyclophilin that binds the zinc finger protein MEP-1 in *C. elegans* (Belfiore et al., 2004). This interaction is essential for the sperm-oocyte switch, though the mechanism through which the MOG-6-MEP-1complex antagonizes *fem-3* is unclear. MOG-6-MEP-1 does not regulate transcription of NOS-3 or FBF but is able to inhibit the expression of a *fem-3*-UTR construct in the soma (Gallegos et al., 1998), suggesting that a direct interaction with *fem-3* mRNA is the most likely mechanism involved.

1.11 *C. briggsae* sex determination

Sex determination mechanisms in different phyla have been shown to be almost completely unrelated. With the exception of a few genes whose homology can be extended to different model systems, most of the genes involved in *Drosophila* and *C. elegans* sex determination appear to be species-specific (Raymond et al., 1998; Raymond et al., 2000). Examining single steps in the evolution of sex-determining systems is not a feasible task when comparing distantly related organisms in which most signs of pathway homology are gone. The analysis of closely related species, on the other hand, is ideal for bringing to light individual modifications in pathway structure (Haag and Doty 2005).

Orthologues of all known *C. elegans* sex determination genes with the exception of *fog-2* (Nayak et al., 2004) are present in *C. briggsae*. A number of these genes have been cloned (de Bono and Hodgkin 1996; Kuwabara 1996b; Hansen and Pilgrim 1998; Streit et al., 1999). Contrasting with genes in pathways such as apoptosis or vulval development, *C. briggsae* sex-determining homologues show poor sequence conservation with their *C. elegans* counterparts (de Bono and Hodgkin 1996; Stothard and Pilgrim 2003). Low sequence similarity is also detected in sex-determining genes between different *Drosophila* and mammalian species, suggesting that proteins involved in sex determination are evolving faster than other proteins

(Walthour and Schaeffer 1994). Moreover, studies have pointed to a role of positive selection in causing the rapid divergence observed in the evolution of these genes (Whitfield et al., 1993; Walthour and Schaeffer 1994). Functional differences between *C. elegans* and *C. briggsae* sex-determining proteins are suggested by two main experiments. The expression of *C. briggsae* sex-determining genes in the respective *C. elegans* mutant backgrounds (e.g. *Cb-fem-2* expression in a *C. elegans fem-2* mutant worm) yields weak or no phenotypic rescue (de Bono and Hodgkin 1996; Stothard et al., 2002). Moreover, the phenotypes of worms submitted to RNAi experiments for sex-determining genes in *C. briggsae* differ in many cases from the correspondent mutant phenotype in *C. elegans* (*table 1*). To investigate to what extent these differences reflect real changes in the *C. briggsae* sex determination pathway and not limitations of the technique is one of the goals of this thesis.

Analysis of *C. briggsae fem-2* exemplifies how the sex determination pathway is changing in *Caenorhabditis* species. *C. briggsae* FEM-2 shows only 63% sequence identity to *C. elegans* FEM-2. Sequence identity reaches 72% in the phosphatase domain located in the carboxyl region of these two proteins (Hansen and Pilgrim 1998). *C. elegans* males that carry a missense *ts* mutation in *fem-2* (*b245*) show feminization of the tail and germline (Kimble et al., 1984). The soma of hermaphrodites homozygous for *fem-2(b245)* is normal, though these worms are sterile at the restrictive temperature due to lack of sperm (*figure 6*). *C. briggsae* FEM-2 is able to completely rescue the somatic and germline defects in *C. elegans fem-2* mutant males, though XX spermatogenesis is still impaired. This functional difference is reproduced when RNAi is performed against *fem-2* in *C. briggsae* (Stothard et al., 2002). If *C. briggsae* FEM-2 had exactly the same functions as *C. elegans* FEM-2 in sex determination, *C. briggsae-fem-2* RNAi should phenocopy the *C. elegans* mutant phenotype. However, while *C. briggsae fem-2* RNAi males show feminization similar to *C. elegans* X0 *fem-2* mutants, hermaphrodites continue to produce sperm and are not sterile (*figure 6*, *table 1*). Thus, unlike *C. elegans*, germline sex determination in *C. briggsae* hermaphrodites may simply not require FEM-2 activity (Hansen and Pilgrim 1998, this work). Recent evidence suggests that far from being limited to *fem-*

2, the mechanisms of germline sex determination in *C. elegans* and *C. briggsae* hermaphrodites are very distinct (Nayak et al., 2004). Phylogenetic data indicates that hermaphroditism evolved independently in *C. elegans* and *C. briggsae*, both of which have a common gonochoristic (male / female species) ancestor (Kiontke et al., 2004). In fact, independent events that led to hermaphrodite development must have occurred at least 10 times in the rhabditid nematodes (Kiontke et al., 2004). Presumably this involved the modulation of a soma-like pathway in the germline of hermaphrodites. If the ability of the XX germline to transiently adopt both sex fates seen in *C. elegans* and *C. briggsae* derived from convergent evolution and not from a common ancestry, the molecular mechanism underlying these fate decisions should be intrinsically different. Indeed, molecular data have shown that *C. briggsae* lacks a *fog-2* orthologue (Nayak et al., 2004). FOG-2 is an essential component of the GLD-1/FOG-2 complex needed to repress *tra-2* mRNA translation in the *C. elegans* XX germline (Clifford et al., 2000, see above). In *C. elegans*, transiently controlling translation of the *tra-2* mRNA in the germline is the key molecular event that allows self-fertility in an otherwise female animal. Furthermore, *C. briggsae* GLD-1 appears to have the opposite function in sex determination than its *C. elegans* homologue. While *C. elegans gld-1* mutant XX animals are females, *C. briggsae gld-1* RNAi hermaphrodites show masculinization of the germline, a phenotype that resembles *mog* mutants (Nayak et al., 2004). In contrast, other non-sex-determining functions of GLD-1, as in controlling meiotic progression in the adult gonad, are conserved between the two species (Nayak et al., 2004). The mechanism that exists in *C. briggsae* to bypass the X:A ratio in the germline and allow spermatogenesis could involve the adoption of new genes or modification of function of known sex determination homologues.

Caution should be taken when concluding that the low level of sequence similarity between *C. elegans* and *C. briggsae* sex-determining genes is reflected in functional changes at the protein level. Tissue-specific limitations of RNAi techniques have been described, notably in the nervous system and germline (Maine 2001; Kennedy et al., 2004). The difference in phenotype between a *C. elegans fem-2* mutant and *C. briggsae fem-2* RNAi may result from inefficient knockdown of *fem-2* transcript levels rather than a true difference in FEM-2 activity. Furthermore, experiments testing the ability of transgenically expressed foreign proteins to rescue specific mutant phenotypes should be interpreted with skepticism. Though sharing a common origin, orthologues of sex-determining genes in *C. elegans* and *C. briggsae* differ in their evolutionary history. The failure to rescue each other's mutant phenotype does not necessarily mean that these proteins have adopted/lost common functions. Co-evolution with target proteins could have preserved the original function in the pathway while resulting in species-specific interactions.

1.12 Isolating *C. briggsae* sex mutants

Accounting for the rapid evolution of sex-determining proteins in *Caenorhabditis* species will ultimately require the isolation of mutant strains affecting sex determination in non-*C. elegans* species. When these strains are available, sequence divergence and protein function can be compared to address how sex determination mechanisms are evolving in nematodes. If co-evolution is masked under sequence divergence, mutations in *C. briggsae* orthologues should result in similar feminizing and masculizing phenotypes to those seen in *C. elegans* mutants, adding further weight to the role of developmental system drift in the evolution of homologous pathways (True and Haag 2001). On the other hand, phenotypic differences could suggest meaningful functional changes and provide evidence for positive selection acting in the evolution of these genes. To unravel the evolutionary steps that shaped the sex determination pathways in *Caenorhabditis*, I have chosen to return to the original genetic screens that started it all with *C. elegans* more than 25 years ago.

1.13 Objectives

The general objectives of this work were to:

1) Isolate and characterize XX masculinizing mutations in *C. briggsae*.
2) Isolate and characterize X0 and XX feminizing mutations in *C. briggsae*.

3) Determine the genetic interactions between the mutant alleles and their relative positions in the *C. briggsae* sex determination pathway.

2.0 Materials and Methods

Strain lists are provided in *table 3, table 5* and *figure 8*. Primers for PCR and sequencing are provided in *table 2*.

2.1 Culture conditions

Unless otherwise mentioned, worms were grown at 20°C on modified NGM agar plates: 5.9g of "worm mix" (55g Tris-Cl, 24g Tris-base, 310g tryptone, 200g NaCl, 800mg cholesterol) and 20g of agar per liter. Plates were seeded with *E. coli* strain OP50 (Brenner 1974).

2.2 Crosses and phenotype analysis

For crosses, a ratio of 1 virgin L4 hermaphrodite to 4 mature males was generally used. Worms were placed on mating plates, with a reduced bacteria-covered area and left to mate for at least 12 hours at 20°C, unless otherwise specified. Successfully inseminated hermaphrodites were identified by checking for a vulva plug (left by *C. briggsae* males during mating) and transferred to new plates. When no other obvious F1 phenotype was expected, the presence of male siblings (around 50% of total worms) was used as an indicator of cross-progeny.

Sex-specific phenotypes for X0 (male tail, one-arm gonad) and XX worms (hermaphrodite tail, vulva, two-arm gonad, oocytes) were initially scored using a Zeiss Stemi SV11 dissecting microscope. Worms selected for DIC microscopy were anesthetized with 0.02% sodium azide in M9 buffer (22mM KH_2PO_4, 42mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$) and analyzed with a Zeiss Axioskop2 compound microscope.

2.3 Mutagenesis and forward screens

A synchronous population of AF16 (wild type) *C. briggsae* worms was obtained by bleaching gravid adults with an alkaline hypochlorite solution (1% NaOCl, 250mM NaOH) that kills hatched worms but not embryos. Eggs were collected and washed in M9 buffer and L1s allowed to hatch overnight at 20°C in

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plates without bacteria. L1s were collected in 5ml of M9 and spread on 6 bacterial plates with an approximate density of 2,000 worms per plate. Worms (P0 generation) were allowed to grow until late L4 stage at room temperature before being harvested from plates and extensively washed with M9 to remove the remaining bacteria. For ENU (N-nitroso-N-ethylurea) mutagenesis, P0 worms were resuspended in 4950µl of M9 and transferred to a 15ml conical tube. 50µl of freshly prepared 100mM ENU solution in 95% ethanol was added to a final ENU concentration of 1mM. For EMS (ethyl methanosulfate) mutagenesis, 20µl of EMS solution in M9 was added to P0 (L4/early adult) worms resuspended in 5ml M9 to a final EMS concentration of 0.05M. Tubes were sealed and left in a rocker at 20°C. After 4 hours, worms were pelleted and the mutagen solution removed. After 4 washes in M9, around 8,000 worms were spread on 10 bacterial plates (~800 worms per plate) and left to grow and lay eggs for 24 hours at 20°C. I allowed the first L1s to hatch before collect F1 eggs by bleaching the mutagenized P0 worms. Approximately 1,000 F1 eggs from each of the 10 P0 plates were spread onto 10 F1 plates and allowed to hatch and grow until L2 stage.

F2 worms (L2 stage) were picked and singled into new bacterial plates. The progeny of these worms were scored for "*tra*" phenotypes by closely checking for the presence of a blunt, intersex tail among the F2 worms grown at 20°C (with the exception of screen #5 when F2 worms were grown at 25°C). I singled a total of 3,746 F1 worms in 10 independent forward screens (2 with EMS and 8 with ENU), representing a total of around 7,500 mutagenized chromosomes (*figure 9*). To check for maternal effect loci (screens #7 and #9), F2 worms were allowed to self and F3 progeny scored for masculinizing phenotypes.

2.4 Brood size analysis

L4 hermaphrodites were singled and grown at 16°C or 20°C. Worms were transferred to new plates every 24 hours until they stopped laying eggs (~4 days at 20°C or 7 days at 16°C). Eggs laid were allowed to hatch and develop into L4 worms before the total number of animals per each plate was counted. Total brood size for a particular strain was calculated as the average number of viable eggs laid by about 12 hermaphrodites.

2.5 Isolating *ts tra* alleles

In *C. elegans*, *tra* alleles generally masculinize the germline, resulting in sterility in homozygous XX worms (Hodgkin and Brenner 1977). Since both *tra* strains isolated in this work were not fertile at 25°C or 20°C (see Results), I tested for temperature sensitivity hoping a homozygous strain could be maintained at 16°C. Heterozygous hermaphrodites for the *tra* alleles were isolated as phenotypically wild type worms at 25°C from F2 plates (where Tra worms were identified) and transferred to 16°C. At this temperature, all F3 worms were phenotypically wild type. F3 hermaphrodites from 5 plates were singled and allowed to lay eggs at 16°C. I then transferred half of these eggs to 25°C. Homozygous Tra worms were identified by selecting for lines that produced 100% *tra* F3 worms at 25°C whereas showing all (for *ed23*, see Results) or partial (for *ed24*, see Results) rescue of F3 worms grown at 16°C. Finally, isolated homozygous *tra-2(ed23ts)* and *tra-3(ed24ts)* were outcrossed 3 times with wild type AF16 worms to get rid of any unlinked secondary mutations and to check for inheritance patterns and maternal effects.

2.6 Linkage mapping of *ed23* and *ed24*

To look for possible gene candidates for *ed23* and *ed24* alleles, we crossed males of these two mutant strains with hermaphrodites of *C. briggsae* marker strains (carrying mutations mapped to different *C. briggsae* linkage groups, *figure 10*). I selected these markers based on the assumption that the *C. briggsae tra-1*, *tra-2* and *tra-3* loci were syntenic to their *C. elegans* homologues on *C. briggsae* chromosomes (C) II, III and IV, respectively (*figure 10*). *tra-2(ed23ts)* males were crossed with *cby-15 (sy5148)* for CII, *unc(sy5422)* and *cby-4(s1272)* for CIII. *tra-3(ed24ts)* males were crossed to *unc(sy5422)* and *cby-7(sy5027)* for CIV. All marker alleles used were recessive. F1 wild type hermaphrodites were singled and F2 progeny scored for wild type, *tra*, marker and double mutant phenotypes. Linkage was tested by performing a X^2 test ($p<0.05$) whenever the data seemed to reject a typical F2 Mendelian ratio.

Linkage was confirmed by checking for absence of Tra phenotypes in the progeny of F2 marker hermaphrodites (e.g. + *cby-15/+ cby-15*).

2.7 Finding the mutation in *tra-2 (ed23)* cDNA

Total RNA was extracted from 3 plates of *tra-2(ed23ts)* worms grown at 16°C. Briefly, worms were washed with M9, suspended in 4ml of Trizol (Gibco) in a conical tube and placed on ice for 1 hour before being spun at top speed for 10 minutes at 4°C. 200µl of chloroform was added to the supernatant, the mixture vortexed and left to sit for 3 minutes. The solution was then spun for 15 minutes at 4°C and the aqueous solution transferred to new tubes. RNA was precipitated by adding 500µl of isopropanol to the solution and incubating it for 10 minutes on ice. A last spin at top speed for 10 minutes at 4°C concentrated the RNA in the bottom of the tube. The pellet was carefully washed with 100µl of 70% ethanol in DEPC water before being air dried and resuspended in 25µl DEPC water. The RNA solution was incubated at 60°C for 10 minutes to ensure complete solubilization. 5µl of the RNA solution was loaded in a 0.7% agarose gel to check for presence of intact bands for ribosomal RNAs before being used for cDNA synthesis.

Approximately 2µl of total RNA was used as template for the first cDNA synthesis reaction with Superscript II Reverse Transcriptase (Invitrogen) and 10 pmol of random (adaptor) primer (Gibco). The reverse transcriptase reaction was performed according to the Invitrogen's Superscript protocol. 1µl of first strand cDNA was used as template for a PCR reaction with primers Cbtra-2RTCF and Cbtra-2RTCR to amplify a 920bp cDNA fragment ("fragment-C"), containing the region corresponded of the end of exon 8, exons 9, 10, 11, 12, 13 and beginning of exon 14 of the *C. briggsae tra-2* gene. The PCR conditions were 95°C 2 minutes (1x); 95°C 30 seconds, 55°C 30 seconds, 72°C 90 seconds (30x); 72°C 3 minutes (1x). The PCR product was gel-purified using Sephaglas BandPrep kit (Amersham) and cloned into pGEM-T (Amersham). Sequencing reactions were performed using Cbtra-2RTCF and Cbtra-2RTCR to cover the 5' and 3' end of fragment C, respectively. I sequenced 10 independent clones of pGEX-fragment C using a Taq:Pfu polymerase mixture of 25:1 to reduced the occurrence of PCR-induced mutations.

RNA extraction, cDNA synthesis, PCR amplification and synthesis of *tra-2* cDNA were also performed using the *C. briggsae* wild type strain (AF16) as control.

2.8 Confirming the mutation in the genomic DNA of *tra-2(ed23ts)*

To rule out a PCR origin for the identified molecular lesion in the cDNA, I sequenced the correspondent genomic region (exon10) of *C. briggsae tra-2* in mutant and wild type worms. The genomic region encompassing *C. briggsae tra-2* was obtained from the *C. briggsae* shotgun library (contig cb25.fpc2454). The *C. briggsae* genome database is available at http://genome.wustl.edu/projects/cbriggsae. I used the available *C. briggsae tra-2* ORF (Wormbase CBG1119, gene 00032357) and an online gene-finding software (http://www.softberry.com/berry.phtml) to determine the putative exon/intron boundaries in the genomic sequence. Sequence primers were designed based on this sequence (*table 2*).

Genomic DNA from *tra-2(ed23ts)* and AF16 strains were isolated from worms grown on 6 rich agarose plates (50mM NaCl, 0.75% peptone, 1.5% agarose, 1mM $CaCl_2$, 1mM $MgSO_4$, 25mM KH_2PO_4, 5μg/ml cholesterol). Worms were collected when plates had almost cleared of bacteria. After extensive washes in 15ml ddH_2O, worms were pelleted down and aliquoted into tubes with no more of 200μl of packed worms per tube. 500μl of lysis buffer (100mM Tris-Cl pH 8.5, 100mM NaCl, 50mM EDTA, 1% SDS, 1% β-mercaptoethanol, 100μg/ml proteinase K) was added to the worm pellet and the solution frozen at –80°C for 30 minutes. The solution was thawed at room temperature and left at 60°C overnight to allow proteolysis to occur. The following day, lysates were extracted twice with 700μl phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with 1.2ml 95% ethanol, gently spooled with a glass rod and washed with 70% ethanol. After being air-dried, DNA was resuspended by placing the rod in 800μl TE overnight at 4°C. 1μl of the extracted genomic DNA was separated in a 0.7%

agarose gel to check for fragment integrity before being used as template for PCR reactions.

I used the primers Cbtra-2GENINCFO that anneals at intron 9 of *C. briggsae tra-2* and Cbtra-2GENINRE that anneals at exon 10 to amplify a 290bp genomic fragment spanning the D587A mutation that had been previously detected in *tra-2* cDNA (see Results). 1 µl of a 1:10 dilution of AF16 and *tra-2(ed23ts)* genomic DNA was used in PCR reactions under the following conditions: 95°C 2 minutes (1x); 95°C 30 seconds, 60°C 30 seconds, 72°C 45 seconds (25x); 72°C 3 minutes (1x). The 290bp genomic fragment was band purified, cloned into pGEX and sequenced using both Cbtra-2GENINCFO and Cbtra-2GENINRE primers. cDNA and gDNA sequences of *C. briggsae tra-2* in mutant and wild type worms were then compared to the locus corresponding to residue 587 of *C. briggsae* TRA-2.

2.9 Complementation crosses for *tra* mutants

2.9.1 Homozygous strains

For *tra-2(ed23ts)* and *tra-3(ed24ts)*, unaffected males were used for crosses with hermaphrodites phenotypically rescued by growth at 16°C. The phenotypes of F1 hermaphrodites at 25°C were checked. Presence of wild type F1 cross-progeny suggested non-allelic mutations whereas *tra* worms indicated allelic mutants.

2.9.2 Balanced strains

Two other non-allelic, non-*ts tra* strains provided by Dr. Eric Haag were used in this study. *tra(nm1)* worms are balanced with *cby(nm4)* (see Results). For *tra(nm1)* complementation experiments, heterozygous *tra + / + cby* hermaphrodites were mated to *tra-2(ed23ts)* and *tra-3(ed24ts)* males. To avoid scoring for F1 hermaphrodites derived from selfing, I first checked for presence of males and absence of Cby worms among F1 animals. At 25°C, presence of 100% wild type hermaphrodites in all cross-progeny analyzed was indicative of non-allelic mutants (e.g. *tra-3/+*; + + / *tra* +) whereas presence of *tra* worms (e.g. *tra-3 + / tra +)* among F1 XX animals indicated allelic mutations.

The *tra(nm2)* strain is balanced with a lethal allele [*let(nm28)*] isolated in the Haag lab. *let/let* worms died as embryos regardless of their phenotypic sex. Therefore, the progeny of the balanced heterozygote is made of wild type balanced hermaphrodites (*tra +/+ let*) and *tra(nm2)* homozygous XX *tra* worms (*tra +/ tra +*). Since the later develops somatically as a male (see Results), it is hard to distinguish cross-progeny males from transformed XX *tra(nm2)* hermaphrodites when *tra(nm2)* heterozygotes are used for crosses. To solve this problem a transgenic dominant marker was used. I inferred from the crosses with *tra(nm1)* that *tra-2(ed23ts)* could not be allelic with *tra(nm2)* (see Results), therefore I concentrated on testing allelism between *tra(nm2)* and *tra-3(ed24)*. Complementation between *tra-3(ed24ts)* and *tra(nm2)* was tested by making a double mutant carrying the *ed24* allele and an integrated *C. elegans rol-6* chromosomal array. *rol-6/rol-6* as well as *rol-6/+* worms are easily identified because of the distinct rolling motion when crawling on the agar surface. In fact, *C. elegans rol-6* can also be used as a marker in *C. briggsae*. Temperature rescued *tra(ed24ts); rol-6* hermaphrodites were heat shocked at 30°C for 4 hours to increase the rate of X-chromosome nondisjunction and male rollers were isolated in the progeny. I then crossed these males (*tra;rol-6*) with *tra + / + let* heterozygous (balanced) hermaphrodites and checked for a progeny where 100% of worms rolled (cross-progeny *rol-6/+*). The prediction was that if *nm2* and *ed24* were allelic mutations, some of the F1 rollers would be *tra* worms (*tra + / tra +; rol-6/+*) whereas presence of only *non-tra* rollers would indicate non-allelic mutations (*tra + / + +; tra/+; rol-6/+*). In the case the latter was true, I tested for the presence of the *nm2* allele in the F1 roller by selfing *non-tra* hermaphrodites and checking the progeny for the presence of *tra(nm2)* transformed XX worms at 16°C (*tra + / tra +*).

2.10 Temperature sensitive period analysis for *ed23*

Temperature sensitive mutants are informative in that they can be used to investigate the time during which the absence of a certain protein product (or the abnormal action of a mutant protein) results in the mutant phenotype (Hirsh and Vanderslice 1976). Thus, *ts* alleles of genes necessary for normal development can indirectly specify periods during development when the activity of the wild type protein is important (Klass et al., 1976).

Because *tra-2(ed23ts)* worms are completely rescued at 16°C (see Results), I decided to characterize the temperature sensitive period (TSP) for the somatic and germline mutant phenotypes involved. Synchronous populations of *tra-2(ed23ts)* worms were obtained by hypochloride treatment of eggs laid by worms grown at 16°C. Eggs were washed in M9 and allowed to hatch overnight at 16°C on plates with no bacteria. In these conditions, larval development arrests at the L1 stage. The following day, L1 worms were washed off the plates and spread onto two sets of 8 bacteria plates (approximately 1000 worms per plate). 8 plates were immediately placed at 25°C (shift-down) whereas the remaining 8 plates were left at 16°C (shift-up). With the exception of one plate of each set, which was left at the original temperatures (25°C and 16°C controls), the other plates were shifted to the restrictive (16°C to 25°C) or permissive (25°C to 16°C) temperatures at gradually later stages of larval development (*figure11*). Worms were allowed to grow to adulthood at the new temperatures and their progeny were scored for specific somatic and germline-related Tra phenotypes.

Somatic effects of *tra-2(ed23ts)* were first addressed by looking at the degree of masculinization of the hermaphrodite tail. Worms in each plate were scored for either a *tra* tail (regardless of the degree of masculinization) or a completely wild type hermaphrodite tail. Therefore, I have qualitatively analyzed the effect of temperature in the adoption of the *tra*-like tail by concentrating on the minimum period necessary to disrupt the wild type developmental program in the female soma. A percentage of "*tra* tails" was calculated for each plate and a curve constructed for both sets of data (shift-up and shift-down).

Germline masculinization caused by *ed23* was assessed by scoring for the presence of eggs inside the hermaphrodite gonad as an indicator of the restoration of fertility (production of sperm and oocyte and somatic structures needed for fertilization). I scored for rescued fertility regardless of the ability of the worm to lay eggs (rescue of vulva, egg-laying phenotypes) or of any other somatic effects of *ed23* not needed for fertility, including tail masculinization. Worms were either fertile (with eggs in gonad) or not. The degree of rescue (brood size) was not taken in consideration. As with the somatic effects of *ed23*, I concentrated on the minimum developmental period during which restoring TRA-2 activity allowed beginning of oogenesis in the germline. The percentage of worms with eggs (fertile) was calculated for each of the 14 plates and curves for each of the set of data were plotted in a graph.

The TSPs for the tail and germline phenotypes, under the limitations of the analysis performed, were determined as the period spanned from the earliest time in shift -down experiments when the phenotypes were first seen until the latest time in shift-up experiments when the phenotypes were seen last (Klass et al., 1976).

2.11 Suppressor screens – *ed23*

The general EMS mutagenesis protocol described above was also used for suppressor screenings. A total of 800,000 EMS mutagenized *tra-2(ed23ts)* chromosomes were scored for suppression of *ed23*-induced sterility (germline phenotype). I decided to concentrate in worms with restored fertility mainly because that was not an expected *tra* suppressor (*sup*) phenotype in *C. elegans* and because I did not find any completely feminized worms (see Results). To avoid redundancy in selecting for suppressors, worms from each of the mutagenized P0 plates were treated separately and F1 plates labeled according to their original parental stock. Plates where eggs could be seen in the bacteria layer were scanned and the fertile *sup* hermaphrodites singled. At the same time, worms that harbored eggs inside the gonad but were unable to lay them were also picked. As expected, suppression of the tail phenotype usually accompanied the germline suppression (though at different degrees), reflecting the dual role in the germline and somatic sex determination pathways of *tra-2* suppressor genes (see Results).

Sup worms (*tra-2;sup*) were singled and left at 20°C until three to four generations had passed and an established strain could be maintained. I initially characterized the general phenotype for hermaphrodites of all *sup* strains at 20°C, 25°C and 16°C to verify the epistatic nature of the *sup* alleles and possible temperature sensitivity. Particular attention was paid to the tail and vulva structures, somatic gonad and germline. To obtain males, young adults from all *sup* strains were heat shocked as described above and the progeny checked for males. When available, *sup* males were tested for fertility and a male/hermaphrodite *sup* strain maintained for use in crosses.

2.12 Genetic analysis of *tra-2(ed23ts) sup* strains

The nature of *sup* alleles was initially tested by:

2.12.1 Backcrossing – suppressor or revertant

Sup hermaphrodites were crossed with *tra-2(ed23ts)* males and the cross-progeny analyzed at 25°C. Presence of *tra* worms indicated that the allele was a true recessive suppressor (*tra-2/tra-2; sup/+*). Absence of *tra* F1 worms suggested an intragenic (revertant) allele or an extragenic, dominant *sup* allele. Feminization of F1 males was then checked and added to the data on inheritance of the *sup* allele. I assumed, based on what is known in *C. elegans*, that mutations in genes that suppress *tra-2* could potentially also feminize the X0 soma and germline. In principle, *sup* alleles that are recessive in suppressing *tra-2(ed23ts)* XX masculinization (*tra-2;sup*) would likewise only have feminizining effects on homozygous males. In addition, I noticed two special cases of inheritance involving partial *tra-2* suppression in *tra-2/tra-2; sup/+* due to a maternal absence effect in m-z+ hermaphrodites and the appearance of F1 feminized males due to a dominant, male-specific effect (haploinsufficiency) (see Results).

2.12.2 Outcrossing – isolating sup alleles.

Sup hermaphrodites were crossed with wild type AF16 males and the F1 and F2 progeny analyzed at 25°C. The recessive or dominant nature of the *sup* alleles in suppressing hermaphrodite masculinization and / or feminizing the male soma and / or germline, was confirmed once more by checking the ratio of wild type F1 (100% wild type hermaphrodites) and F2 (3 *tra* : 16 wild type) animals at 25°C. In *C. elegans* Fem worms, the XX feminization phenotype seen in *fem/fem* animals is identical in *tra-3;fem* (or *tra-2;fem*) double mutants, revealing the complete suppression by epistasis nature of these alleles (Hodgkin 1986). Since *sup* hermaphrodites did not seem to have the same effect on XX worms (see Results), I decided to check whether in *C. briggsae*, the ability of these alleles to feminize the hermaphrodite was somehow hidden by the *tra-2* mutation. Though that was not expected based on the genetic interactions seen in *C. elegans tra-2* and *fem* genes, I hypothesized that some *sup* alleles only incompletely suppress *ed23*, explaining why spermatogenesis still occurred in *sup* strains. For that purpose, the F2 progeny of outcrossed *sup* hermaphrodites was carefully checked for specific (including feminized XX) *sup* phenotypes (*+;sup*) not previously seen in the double mutant (*tra-2;sup*).

To investigate possible maternal effect of *sup* alleles, 15 XX F2 worms from outcrossed *sup* hermaphrodites were randomly picked and singled. The F3 progeny in these plates was checked for any feminizing phenotype (partial *tra-2* suppression) absent in the F2 generation. In addition, crosses between F1 males and hermaphrodites were performed whenever F1 males from outcrossed hermaphrodites were fertile. Similarly, F2 males from these crosses were check for feminizing phenotypes (e.g. oocyte-like cells in the gonad, attempt to make a vulva and abnormal male tail).

2.13 Suppressor screens – *ed24*

Screens for suppressor alleles of *tra(ed24ts)* were performed following the same protocol described above for *tra-2(ed23ts)*. A total of 240,000 EMS mutagenized *tra(ed24ts)* haploid genomes were scored for suppression of somatic and germline masculinization in three independent screens. The genetic analysis of *tra;sup* strains was identical to the one described above for *tra-2;sup* strains.

2.14 *fem-2(nm27)* genotyping

A molecular strategy to genotype *fem-2(nm27)* mutant hermaphrodites was developed in the Haag lab to overcome the lack of a characteristic hermaphrodite mutant phenotype. Primers EH21 and EH22 anneal inside a genomic region in the 3' end of *C. briggsae fem-2* ORF that is deleted in *fem-2(nm27)* worms and therefore can be used to discriminate homozygous *fem-2* mutants (*figure 6)*. In PCR reactions with EH21 and EH22, genomic DNA templates from *fem-2/+* or +/+ worms yielded a 560bp fragment while *fem-2/fem-2* DNA templates did not. I genotyped individual worms to check for homozygosity of the deletion in the *fem-2* locus. Single worms clean of bacteria were resuspended in 2.5μl of lysis buffer (50mM KCl, 10mM Tris pH 8.2, 2.4mM $MgCl_2$, 0.45% NP40, 0.45% Tween-20, 0.01% gelatin, 2.5mg/ml

Proteinase K) and frozen at -80°C for 30 minutes. Worms were next lysed at 60°C for 1 hour and the reaction stopped at 95°C for 15 minutes. Partially extracted DNAs from single worms were used as template for PCR reactions with the EH21 and EH22 primers. PCR conditions were as follow: 95°C 2 minutes (1x); 95°C 30 seconds, 60°C 30 seconds, 72°C 60 seconds (35x); 72°C 3 minutes (1x).

2.15 Complementation of *ed23* and *ed24 sup* alleles

2.15.1 ed23 suppressors

Hermaphrodites of 21 *sup* strains were used for complementation crosses. Due to the absence of any other mutant *sup* phenotypes in XX worms, I used the ability of *sup* alleles to suppress *ed23* to test for allelic mutants. Considering the recessive nature of *sup* alleles, non-allelic mutations should fail to suppress the *tra-2* mutation (e.g. *tra-2/tra-2;sup1/+;+/sup2*). I concentrated on identifying members of two non-allelic complementation groups; a *fem-2* group using strain DP369 [*tra-2(ed23) cby-15(sy5248); fem-2(nm27)*] and a *fem-A* (see below) group represented by the *sup* allele in the strain DP373 [*tra-2(ed23ts);fem-A(ed31)*]. I selected this strategy because these alleles were among the few that did not completely sterilize homozygous or heterozygous males, allowing them to be used in crosses.

Males from the DP373 strain were crossed with other *sup* hermaphrodites (*tra-2;sup*) and F1 XX animals scored for Tra phenotypes at 20°C. The presence of *tra* worms in the F1 cross-progeny indicated non-allelic mutations. Progeny with 100% wild type F1 hermaphrodites suggested allelic mutations (e.g. suppression of Tra phenotypes in a *tra-2/tra-2;sup1/sup2* genotype).

To identify members of the *fem-2* allelic group I crossed hermaphrodites of the 21 *sup* strains (*tra-2;sup*) with *tra-2 cby-15/tra-2 +;fem-2/+* males. The *cby-15* allele served as a marker in *cis* with *ed23* on chromosome II useful in identifying *tra* genotypes at 16°C, when Tra phenotypes are absent. Cross-progeny were raised at 25°C. In theory, non-allelic mutants should produce 100% *tra* hermaphrodites in F1 (e.g. *tra-2 +/tra-2 +;fem-2/+;sup/+*) while some wild type (suppressed) F1 hermaphrodites would be seen in the case of allelic mutants (e.g. *tra-2 +/tra-2 +; fem-2/sup*). However, because *fem-2/+* males only produce around 20 sperm cells before becoming sterile (see Results), the F1 progeny invariably contained worms that resulted from selfing of the *sup* hermaphrodite mother used in the cross. Thus, F1 wild type hermaphrodites cannot be always taken as indicative of allelism because of mixed cross and self-progeny. Because *tra-2;sup* XX worms cannot always be phenotypically distinguished from wild type hermaphrodites (complete suppression), I relied on the analysis of the F2 generation to distinguish between non-allelic and allelic cases. I used the *cby-15* allele in the male as a marker for F1 genotypes derived from cross-fertilization. About 20 F1 wild type worms were singled in every complementation cross and the individual F2 progeny analyzed for *cby* animals. The presence of F2 Cby worms indicated complementation of the *sup* and *fem-2* alleles (original F1: *tra-2 cby-15/tra-2 cby-15; fem-2/sup* –wild type phenotype due to complementation). F1s derived from selfing of the original *sup* hermaphrodites would never inherit the *cby-15* allele from the male and only produced *sup* F2 worms (*tra-2 +/tra-2 +; sup/sup* – "wild type" phenotype due to complete suppression). I concluded that the Tra phenotype seen in the F1 progeny of these lines were all cross-progeny worms and therefore the *fem-2* and *sup* mutations could not be allelic (e.g. *tra-2 +/ tra-2 +; fem-2/+; sup/+*). Conversely, allelic mutants showed some wild type F1s that when selfed produced F2 Cby worms, as expected (*tra-2 cby-15;sup*).

2.15.2 *ed24 suppressors*

Fertile males from the *tra-3;sup* strain, DP375, were used in crosses with hermaphrodites of the other two isolated *sup* strains (DP396, DP397). F1 progeny were analyzed at 25°C. *tra* F1 worms indicated non-allelic mutations, all wild type F1 progeny supported an allelic nature for the alleles. The *sup* allele in DP375 is referred hereon as *fem-C(ed32)*.

2.16 Isolation and characterization of *fem-B(ed30)*

To isolate the suppressor allele in one *tra-2(ed23ts) sup* strain (DP374) I performed outcrosses and selected for lines that lacked *tra* worms (*ed23* phenotype) while showing the characteristic dominant feminization phenotype of *sup* males (see Results). *Sup* worms (*tra-2;sup*) were grown at 16°C and mated to wild type AF16 males. 8 F2 wild type hermaphrodites originating from a F1 (*tra-2/+; sup/+*) worm were singled and grown at 25°C. The F3 progeny from 2 F2 hermaphrodites that produced *tra* worms (e.g. F2 mother *tra-2/+; sup/+*) were discarded. 5 wild type F3 hermaphrodites from the remaining 4 F2 lines were crossed with *tra-2(ed23ts)* males and the cross-progeny that showed: a) no *tra* hermaphrodites and b) 100% feminization of males (*+;sup*) isolated (DP366). Based on its predicted position in the pathway (downstream of *tra-2* and *tra-3*) and despite the lack of a typical *C. elegans* Fem phenotype (see Results), I refer to this gene and its *sup* allele as *fem-B(ed30)*.

A *fem-B(ed30);mih-3(s1290)* strain (DP367) was constructed to allow scoring for homozygous males. The *mih-3* allele increases the frequency of X-chromosome nondisjunction in the hermaphrodite meiotic cells, similar to *him* (high incidence of males) mutations in *C. elegans*. *mih-3/mih-3* males were crossed with *fem-B(ed30)* hermaphrodites and the F1 XX worms selfed. 30 F2 lines that produced F3 males were analyzed. Progeny that showed 100% of males feminized were selected (*fem-B;mih-3*). Because the feminization of *ed30* males is dominant (see Results), hermaphrodites from these lines were crossed with AF16 X0 worms to confirm the absence of wild type males in the cross-progeny (*fem-B/fem-B*). *fem-B(ed30)* males were further grown at 16°C, 20°C and 25°C to test for temperature sensitivity of the feminization phenotype.

The isolated *ed30* allele was re-introduced into a *tra-2(ed23)* genotype (DP370) to test whether it really was the *sup* allele in *tra-2;fem-B* worms or simply a secondary feminizing mutation unrelated to *tra-2* suppression that fortuitously accumulated in the mutagenesis experiment. The crosses were made at 16°C and the *tra-2(ed23ts)* allele followed using *cby-15* as a linked marker. *tra-2 cby-15/+ +; +/+* males were crossed with *fem-B(ed30)* hermaphrodites (*+ +;fem-B*) and 15 F1 hermaphrodites singled. Half of the F1 plates produced F2 Cby worms (F1 *tra-2 cby-15/+ +; fem-B/+*). Cby hermaphrodites were grown at 16°C, shifted to lay eggs at 20°C and analyzed for their phenotype. I looked for the presence of Cby worms with a *tra-2;fem-B*-like phenotype at 20°C phenotype (see Results) as the indication that *fem-B(ed30)* was the original *sup* allele in *tra-2;fem-B* worms.

2.17 Shift-up experiments of *tra-2(ed23ts);fem-B(ed30)* worms

For the embryonic and larval shift-up experiments, *tra-2;fem-B* and *tra-2* hermaphrodites were grown at 16°C and the embryo or larval progeny shifted to 25°C at different developmental time points. Embryos were shifted to 25°C at 2-cell ($N_{tra\text{-}2}=5$ $N_{tra\text{-}2;fem\text{-}B}=4$), "comma" ($N_{tra\text{-}2}=5$,$N_{tra\text{-}2;fem\text{-}B}=7$) and "pretzel" ($N_{tra\text{-}2}=5$,$N_{tra\text{-}2;fem\text{-}B}=8$) stages, whereas larvae were shifted at L1($N_{tra\text{-}2}=50$ $N_{tra\text{-}2;fem\text{-}B}=50$), L2 ($N_{tra\text{-}2}=50$ $N_{tra\text{-}2;fem\text{-}B}=50$) and L3 ($N_{tra\text{-}2}=50$ $N_{tra\text{-}2;fem\text{-}B}=50$) stages. Embryos were staged by light microscopy and larval stages by synchronizing worms as described above for TSP experiments. The tail and germline phenotypes of adult worms grown at 25°C were analyzed.

The effect of temperature on oocytes was investigated through shift-up experiments performed by transferring a young adult *tra-2;fem-B* hermaphrodite grown at 16°C to 20°C. These worms were fertile egg-laying hermaphrodites. Eggs laid hourly for 8 consecutive hours (N=2 eggs for every hour) were collected in a separate plate and left at 20°C where they hatched and developed into adult worms. Based on what is known of the *C. elegans* oocyte maturation program, eight hours at 20°C should account for germline events such as nuclear migration, nuclear breakdown, cortical rearrangement, ovulation and fertilization (Kemphues 1997). I checked for the tail and germline phenotypes of shifted adult worms and compared them to *tra-2;fem-B* worms grown at 16°C and 20°C to determine the effect of temperature on the spectrum of suppression.

2.18 Maternal effect of *fem-B* and *fem-2*

Many aspects of *C. elegans* embryonic and larval development are completely or partially dependent on the expression of maternal mRNAs or protein inherited from the oocyte. In *C. elegans*, maternally contributed *fem* mRNAs are able to make up for the lack of zygotic expression (m+z-) (Hodgkin 1986). For example, *C. elegans fem-2/fem-2* animals derived from selfing of *fem-2/+* mothers have a mutant genome (lack of zygote expression of the *fem-2* locus) but developed from a wild type oocyte (or m+z-). These worms, different from m-z- worms, are hermaphrodites and not females. Thus, in cases of maternal rescue, the phenotype of the progeny entirely depends on the mother's genotype expressed in the oocyte. I decided to investigate the role of maternal and zygotic expression of the *C. briggsae tra-2;sup* genes. Because the mutant alleles for these genes do not cause a specific XX mutant phenotype other than suppressing *tra-2(ed23ts)*, (m-z- worms are as "wild type-looking" as m+z+), I checked for the existence of a maternal *absence* effect in suppressing the germline and somatic defects of *tra-2(ed23ts)* in double mutants (*tra-2;sup*). Maternal absence is just as informative in terms of concluding for a role of maternal mRNA or protein to the embryo/larva development as the classic maternal rescue analysis. For instance, *C. elegans fem-3/+* males from *fem-3/fem-3* mothers (m-z+) are incompletely masculinized and even transformed to females at times. Presumably, maternal contribution of *fem-3+* is needed, even in the presence of a zygotically expressed wild type product, in order to completely masculinize the worm (Hodgkin 1986).

If maternally provided C. *briggsae fem-2+* and *fem-B+* mRNA play a role in male development, FEM activity in *tra-2;fem-2* and *tra-2;fem-B* embryos/larvae from *fem-2/+* and *fem-B/+* mothers (m+z-) should result in no suppression of the Tra phenotype. Consequently, I would expect some degree of somatic/germline suppression to occur in worms that inherited the mutant *fem-2* or *fem-B* mRNAs from their mother (m-z+). However, if no maternal RNA is needed and zygotic expression of the *fem* loci is responsible for all (or enough) protein activity during development, then m+z- *tra-2;fem-2* and *tra-2;fem-B* worms should be as suppressed as the worms in which no expression occurs at all (m-z-). This being true, the lack of maternal *fem* RNA would have no effect on the *tra-2* phenotype and m-z+ hermaphrodites should ultimately develop to become *tra* adults.

2.18.1 fem-B(ed30)

m-z+ hermaphrodites were obtained by crossing *tra-2 cby-15;fem-B* XX with *tra-2* males (*tra-2 +; +*) at 16°C and growing the F1 cross-progeny at 25°C. I next selfed m-z+ (*tra-2 cby-15/tra-2 +; fem-B/+*) hermaphrodites grown at 16°C to isolate m+z- worms (*tra-2 cby-15;fem-B*). The phenotype of m+z- worms was compared with *tra-2;fem-B* (m-z-) hermaphrodites grown at 25°C. As a control for lack of suppression, I made m+z+ (e.g. *tra-2 +; +*) worms by crossing *tra-2 cby-15/tra-2 +; fem-B/+* (m-z+) hermaphrodites with *tra-2* males.

2.18.2 fem-2(nm27)

m-z+ hermaphrodites (*tra-2 cby-15/tra-2 +; fem-2/+*) were obtained by crossing *tra-2 cby-15; fem-2* XX worms with *tra-2* males (*tra-2 +; +*) at 16°C. As described above for *fem-B(ed30)*, m-z+ XX worms grown at 16°C were selfed and crossed with *tra-2* males to obtain m+z- and m+z+ worms, respectively. Phenotypes of adult hermaphrodites and *tra-2;fem-2* (m-z-) double mutants were compared to define the level of suppression.

2.19 X-linkage analysis of *fem-B(ed30)*

To check whether the male-specific dominant effect of *ed30* feminization was due to an X-chromosome location or haploinsufficiency of the *fem-B* locus, I used *cby-3*, a *C. briggsae* X-chromosome marker, and tested linkage to *ed30*.

fem-B(ed30) hermaphrodites were crossed with *cby-3(bd101);mih-1(bd102)* males and F1 cross-progeny XX worms selfed. F2 Cby worms (either $X^{fem\text{-}B,cby\text{-}3}X$, if *fem-B* is X-linked or $X^{cby\text{-}3}X$; *fem-B/+*, if autosomal) were crossed to wild type (AF16) males and F3 males analyzed for the germline feminization (Fem-B phenotype). If *fem-B* is an X-linked gene, mated F2 Cby worms (F2 $X^{fem\text{-}B+,cby\text{-}3}X$) should never produced mutant X0 worms when crossed to wild type males. Conversely, feminized *fem-B* males would appear in the cross-progeny in the case of an autosomal location (e.g. F2 $X^{cby\text{-}3}X^{cby\text{-}3}$;*fem-B/+*).

2.20 Haploinsufficiency effect in *fem-B(ed30)* and *fem-2(nm27)* males

To explain the dominant masculinization of *fem-B* and *fem-2* in X0 worms, I looked at heterozygous males in different maternal mRNA contribution backgrounds.

2.20.1 fem-B(ed30)

fem-B(ed30) hermaphrodites were crossed with wild type AF16 males and the germline of *fem-B/+* F1 males (m-z+) analyzed. Subsequently, *fem-B/+* hermaphrodites were crossed with AF16 males and the male progeny obtained (m+z+; 50% *fem-B/+*, 50% +/+) was scored for germline feminization. The reciprocal cross (males *fem-B/+* x wild type hermaphrodites) could not be done due to sterility of *fem-B/+* males.

2.20.2 fem-2(nm27)

fem-2(nm27) hermaphrodites were crossed with wild type AF16 males and F1 males (m-z+) checked for feminization phenotypes. F1 *fem-2/+* hermaphrodites and males were reciprocally crossed to wild type AF16 worms. Males in the cross-progeny (m+z+) were also checked for signs of feminization.

2.21 Constructing double mutants – epistasis analysis

2.21.1 tra-2(ed23ts) – tra(ed24ts)

I looked for the double mutant by scoring for Cby worms (in *cis* with *ed23*) that produced *tra* hermaphrodites at 16°C (*ed24*-specific phenotype). *tra-2 cby-15* hermaphrodites were crossed with *tra(ed24ts)* males at 16°C. F2 Cby worms derived from selfing of F1 hermaphrodites (*tra-2 cby-15/+ +; tra/+*) were singled at 16°C. Rescued hermaphrodites from 4 different F3 generations that showed *cby-tra* worms at 16°C were next crossed with *tra(ed24ts)* and *tra-2(ed23ts)* males and the cross-progeny grown at 25°C to confirm the presence of both *tra* alleles in the genotype. I selected for lines that showed 100% *tra* XX worms in the cross-progeny in both crosses at 25°C. The homozygous strain was maintained at 16°C. Males were obtained from sporadic nondisjunction events.

2.21.2 tra-1(nm2) – tra-2(ed23ts)

Considering that *nm1*, *ed23* and *ed24* are fairly strong alleles (see Discussion) and the majority of the feminizing signal in *C. briggsae* soma is presumably mediated through TRA-1 activity, the observed differences in Tra phenotypes can be used to reveal a hierarchy of feminizing genes in this species. In short, if *C. briggsae tra-1* is downstream of *tra-2* and *tra-3* in the pathway, the strong phenotype seen in *nm2* XX mutants should override any feminizing signal remaining in *tra-2(ed23ts)* and *tra(ed24ts)* worms. I checked the phenotype of *cby* XX worms at 16°C in the cross below to check whether the temperature rescue of *ed23* phenotypes was epistatic or hypostatic to *nm2*. Assuming that all three *tra* genes act in the same pathway and that *tra-1* is downstream of *tra-2*, Cby worms with a typical *tra-1* phenotype should appear at 16°C.

tra-1 balanced hermaphrodites (*tra-1 +/+ let*) were crossed with *tra-2 cby-15/+* + males. F1 XX worms were singled and F2 lines scored for presence of Cby-Tra-1 transformed XX worms at 16°C (*tra-1 +; tra-2 cby-15*). Appearance of Cby-Tra-1 worms at 16°C was interpreted as the triple mutant phenotype (*tra-1;tra-2 cby-15*).

2.21.3 tra-1(nm2) – tra(ed24ts)

Since I lacked a phenotypic marked in *cis* with e*d24*, the double *tra* mutant was observed by checking for the appearance of *tra-1*-like XX transformed worms in previously selected *tra(ed24ts)* homozygous background.

tra-1 balanced hermaphrodites (*tra-1 +/+ let*) were crossed with *tra(ed24ts)* males. F1 hermaphrodites (*tra/+; + +/tra-1* + or *tra/+; + +/+ let*) were crossed with *tra(ed24ts)* males once more and 50 F2 XX worms were singled. F3 worms were grown at 16°C and hermaphrodites from 8 different plates that showed *tra (ed24)*-like and *tra-1*-like worms were again crossed with *tra(ed24ts)* males. The phenotype of the cross-progeny grown at 25°C was scored. I selected lines that showed *tra(ed24ts)*-like but not wild type hermaphrodites at 25°C, indicating homozygosis for the *ed24* locus. The presence of the sterile *tra-1*-transformed XX phenotypes in some of these lines was interpreted as the double mutant phenotype (mother: *tra/tra; tra-1 +/+ let*). Because Tra-1 worms are sterile and I did not select for the *let* balancer, the lines could not be maintained.

2.21.4 tra-2(ed23ts) – fem-2(nm27)

Our working hypothesis was that like in *C. elegans*, *fem-2* is downstream of *tra-2* and *tra(ed24ts)* (see below) in the *C. briggsae* sex determination pathway. Thus, the strategy to isolate the *tra-2;fem-2* double mutant was based on the assumed suppression of *tra-2(ed23ts)* phenotype at the restrictive temperature. Crosses were initially done at 16°C using *cby-15* as a phenotypic marker for *ed23*.

fem-2 hermaphrodites were crossed with *tra-2 cby-15/tra-2* + males. F1 were singled and F2 Cby worms selected. *Cby* lines were allowed to expand at 16°C. About

10 F3 eggs were transferred from each F2 line to 25°C. Three of these lines showed fertile *cby* F3 hermaphrodites (suppressed *tra-2* phenotype) in the cross-progeny. Adult F3 worms from these three lines were crossed with *tra-2(ed23)* males. Cross-progeny with 100% *tra* worms at 25°C indicated that suppression of the Tra phenotype seen in these lines was due to presence of the *fem* allele (*tra-2 cby-15; fem-2*) and not to a recombinant + *cby-15* chromosome.

2.21.5 tra(ed24ts) – fem-2(nm27)

Before isolating the double mutant, I initially characterized whether *fem-2* was epistatic to *tra(ed24ts)* by checking the ability of the *nm27* allele to reduce the overall number of lines expected to produce *tra* worms in the cross described below.

fem-2 hermaphrodites were crossed with *tra(ed24ts)* males and *tra/+; fem-2/+* F1 males backcrossed with *fem-2* XX worms. 36 cross-progeny hermaphrodites were singled and the presence or absence of *tra* worms in the progeny checked. In the case of *tra(ed24ts)* being epistatic to *fem-2*, half of phenotypic hermaphrodites (XX or *fem-2* X0 transformed males) from the backcross-progeny should produce *tra* worms when selfed (backcross-progeny *tra/+; fem-2/+* XX, *tra/+; fem-2/fem-2* XX and X0) while the rest should produce only wild type worms (backcross-progeny *+/+; fem-2/+* XX, *+/+;fem-2/fem-2* XX and X0). Conversely, a scenario with *fem-2* downstream of *tra(ed24ts)* predicted that only 1/5 of the backcross lines should show *tra* worms in their self-progeny *(tra/+; fem-2/+* XX) whereas all other genotypes, homozygous for *fem-2(nm27)* and/or lacking the *tra (ed24ts)* allele should result in completely wild type progeny (*+/+; fem-2/+* XX, *tra/+; fem-2/fem-2* XX and X0, *+/+; fem-2/fem-2* XX and X0). Of 36 hermaphrodites isolated from the backcross-progeny, 29 produced only wild type worms when selfed while *tra* worms were only seen in 7 lines.

Lines producing *fem-2;tra* double mutants were identified among the 29 lines lacking *tras* (see above) by crossing hermaphrodites to *tra(ed24ts)* males and checking for plates with 100% *tra* worms in the cross-progeny grown at 25°C. As with *tra-2;fem-2* worms, *tra(ed24ts);fem-2* are fertile hermaphrodites that can be maintain at 25°C.

2.21.6 tra(ed24ts) – fem-B(ed30)

Given the position of *tra-2* and *tra(ed24ts)* in the *C. elegans* sex determination pathway I assumed that *fem-B(ed30)*, isolated as a suppressor of *tra-2(ed23ts)*, would similarly suppress *tra(ed24ts)*. The strategy to isolate the *tra(ed24ts);fem-B* double mutant was based on the assumption that this worm would adopt the characteristic germline suppression phenotype seen in *tra-2;fem-B* hermaphrodites (see Results) over the sterile *tra-2* phenotype. I therefore crossed *fem-B(ed30)* hermaphrodites with *tra(ed24ts)* males, singled F1 XX worms (*tra/+;fem-B/+*) and looked for *tra-2;fem-B*-like worms in the F2 progeny at 20°C.

2.21.7 tra-2(ed23ts) – fem-C (ed32)

Similarly to the case with above, I hypothesized that a *tra(ed24ts) sup* allele would likely suppress *tra-2* mutations. Since isolating a *+/+;fem-C/fem-C* strain was complicated because of the similarity of XX and X0 with wild type phenotypes (see Results), I opted to introduce *tra-2(ed23ts)* into *tra(ed24ts);fem-C* worms and analyze the triple mutant phenotype.

tra-2 cby-15;tra-3 hermaphrodites grown at 16°C were crossed with *tra-3;sup* males. F1 worms (*tra-2 cby-15/+ +; tra/tra; fem-C/+*) were singled and the phenotype of F2 *cby* hermaphrodites analyzed at 20°C. The absence of wild type worms was indicative of lack of suppression in *tra-2 cby-15;fem-C* worms.

Epistasis analysis between *tra-1(nm2)* and *fem* alleles is currently being performed by our collaborators in Dr. Haag's lab.

2.22 Immunostaining of dissected gonads

For reference, a more comprehensive gonad dissection protocol is described by Francis et al., 1995. Briefly, adult hermaphrodites and male worms were put in an unseeded modified NGM plate to clean from bacteria and then suspended in 100μl of 0.2mM Levamisole in PBS on a glass slide (Fluoroslide – Erie Scientific). As paralysis started, worms were decapitated using two 25 gauge syringe needles. This process results in the extrusion of at least the anterior gonad arm in the hermaphrodite and the complete male gonad, both of which remain attached to the worm carcass. The excess of liquid was drained and worm carcasses fixed with ice-cold 100% methanol for 5 minutes at room temperature. After 3 subsequent washes in PBS-T (0.1% Tween 20 in PBS), carcasses were blocked at 4°C overnight with 1mg/ml BSA in PBS-T. First antibody dilutions (anti-GLD-1 antibody: 1:100; anti-SPE56 antibody: 1:50) were made in blocking solution. I used a polyclonal rabbit anti-*C. elegans* GLD-1 antibody (Jones et al., 1996) and a monoclonal mouse anti-*C. elegans* SPE56 antibody (ascite #2) (Ward et al., 1986) to characterize germ cell progression through meiotic prophase and presence of differentiating spermatocytes/ sperm cells, respectively. After two hours incubation at room temperature, carcasses were washed three times in PBS-T and incubated with 1:500 dilutions in PBS-T of anti-rabbit IgG conjugated to Alexa 546 (for GLD-1 staining) (Molecular probes) or anti-mouse IgG conjugated to Alexa 488 (for SPE56 staining). Carcasses were next washed again in PBS-T and briefly stained with 0.1μg/ml 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) to check nuclear morphology of germ cells. After a last wash in PBS-T, slides were mounted and observed under a UV-microscope.

3.0 Results

3.1 Masculinizing mutations

3.1.1 tra-A(ed23ts)

3.1.1.a) Isolation

The progeny of 3,746 F1 hermaphrodite worms (~7,500 haploid chromosomes) were scored for intersex phenotypes (incomplete transformation of hermaphrodites into males) at 20°C in 10 independent mutagenesis screens (*figure 9*). Mutant hermaphrodites that showed abnormal somatic masculinization, in particular incomplete formation of male tail (absent bursa, vestigial rays and malformed spicule), were isolated from the progeny of one such F1 worms in screen #3. These worms showed the classic somatic intersex phenotypes that are characteristic of *C. elegans tra-2* and *tra-3* null mutants (Hodgkin & Brenner 1977); a blunt tail, one-arm gonad and incompletely formed vulva. The germline did not produce oocytes and though worms made sperm, the somatic transformation into males wasn't complete enough to support mating (due to absence of male mating behavior and intersex tail structure). Hence, worms were sterile due to inability to self or cross-fertilize other hermaphrodites (*figure 12*). Based on the similarity to *C. elegans tra* mutants, I initially named this gene and its mutant allele; *tra-A (ed23)*. Upon further testing, *ed23* was revealed to be a *ts* allele. *tra-A* hermaphrodites grown at 16°C (permissive temperature) are completely wild type whereas growth at 25°C (restrictive temperature) yields *tra* worms with complete penetrance. Lowering the temperature rescued not only the somatic defects in the tail and vulva but also the germline sperm-oocyte switch (*figure 12*). The brood size of worms grown at the permissive temperature was comparable to wild type levels (*figure 13*). As observed in *C. elegans tra* mutants, homozygous *tra-A* males grown in both temperatures were not affected and could be normally used for crosses.

3.1.1.b) Inheritance of *ed23*

Backcrossing *tra-A* males to wild type AF16 hermaphrodites or *tra-A* hermaphrodites (grown at16°C) to wild type males resulted in 100% wild type F1 worms. *tra* worms re-appeared in 25% in the progeny of selfed F1 hermaphrodites (m+z-). The lack of F1 mutant worms and the ratio of mutant F2 animals indicate that *ed23* is an autosomal recessive allele and the *tra-A* locus does not have a significant maternal effect (*figure 14*).

3.1.1.c) Linkage mapping

Because of the similarity in phenotypes of *tra-A* and *C. elegans tra-2* mutants, I hypothesized that *ed23* was a *C. briggsae tra-2* allele. Synteny with *C. elegans* genes predicts *C. briggsae tra-2* to be located on chromosome II (CII). 846 F2 XX worms from selfed hermaphrodite heterozygous for *cby-15*, a Linkage Group II marker, and *tra-A* resulted in 480 wild type, 161 Tra worms, 205 Cby worms but no Cby-Tra worms. Indeed, the *ed23* phenotype is linked to *cby-15*, proving that the *tra-A* locus maps to CII (*figure 10*).

3.1.1.d) Molecular Lesion

To prove that *ed23* is a *C. briggsae tra-2* allele and further characterize the nature of the mutation, I sequenced the *C. briggsae tra-2* cDNA. An A to C transversion (A1759C) was present on exon 10 of the *tra-2* cDNA sequence as well as in the genomic DNA but not in the wild type *tra-2* sequences. This missense mutation predicts the substitution of an aspartic acid in the wild type protein for an alanine (D587A) in the extracellular amino domain of TRA-2 (*figures 15 ,16*). Thus, there is genetic and molecular evidence to support that the *tra-A* gene is in fact *C. briggsae tra-2,* and it will be referred as such for the remaining of this thesis.

3.1.2 tra-B(ed24ts)

3.1.2.a) Isolation

A second masculinizing allele, *ed24*, was isolated in the same way as *ed23* in screen#4. *ed24* defines another *tra* gene (see below), initially referred to as *tra-B*. Phenotypically, *tra-B* worms are very similar to *tra-A*. Hermaphrodites are generally smaller than wild type worms, have incomplete (snub) tail and sperm present in the posterior end of a somatically male gonad. X0 worms are not affected (*figure 12*). Furthermore, *ed24* is also a *ts* allele. However, not 100% of worms are rescued at 16°C, and 30 to 40% of *tra-B* XX animals grown at 16°C are not rescued and develop as *tra*. Therefore, the *tra-B* strain differs from *tra-2(ed23ts)* in that at 16°C, *tra* worms are only present in the former (*figure 13*).

3.1.2.b) Inheritance of *ed24*

Similar to *tra-A(ed23)*, F1 and F2 progeny analysis from backcrosses using *tra-B* males and AF16 hermaphrodites, suggested an autosomal recessive nature for *ed24*. In addition, maternal *tra-B* contribution of mRNA or protein does not seem to have an effect on *tra-B(ed24)* worms, indicating that no maternal effect exists for this locus.

3.1.2.c) Linkage mapping

Complementation studies indicated that *ed23* and *ed24* are alleles of two different genes (see Material and Methods). I decided to test if *ed24* was a *C. briggsae tra-3* allele based on the lack of complementation with *tra-1* and *tra-2* alleles (see below). Linkage analysis using *ed24* and a *cby-7*, a Linkage Group IV marker, supported a CIV location for *tra-B*. Selfing of heterozygous XX worms for *cby-7* and *tra-B* resulted in 126 wild type, 61 Cby, 51 Tra and 1 Cby-Tra worm of a total of 239 scored F2 animals. In addition, small nucleotide polymorphism analysis has also mapped *ed24* to chromosome IV (Eric Haag, personal communication). Based on synteny of *C. briggsae* and *C. elegans* genes, *tra-3* is the only *C. briggsae tra* homologue expected to map to CIV (*figure 10*). Though confirmation of the molecular lesion in *tra-B(ed24)* is necessary, the genetic data so far supports the view that *ed24* is in fact an allele of *C. briggsae tra-3*. Therefore, it will referred hereon as *tra-3(ed24ts)*.

3.1.3 Complementation of tra alleles

3.1.3.a) *nm1*and *nm2*

Two other *C. briggsae tra* alleles, provided by Dr. Eric Haag (University of Maryland), were also included in the genetic analysis of *tra* alleles. *tra (nm2)* and *tra (nm1)* are autosomal recessive alleles isolated in forward screens for *C. briggsae* masculinizing genes in the Haag lab. Both alleles result in sterile *tra* worms at all temperatures tested and for that reason have to be maintained as balanced strains. The overall phenotype and degree of hermaphrodite masculinization caused by *nm1* is similar to that noticed for *tra-2(ed23ts)* and *tra-3(ed24ts)*. *nm2*, on the other hand results in complete somatic transformation of hermaphrodites into males (*figure 17*). XX transformed worms not only develop all male tail structures but also display male-specific mating behavior, suggesting that the neuronal wiring that control sex-specific behavior has also been switched (Trent et al., 1983). However, the germline of the adult worms ultimately adopts a hermaphrodite fate, producing oocyte-like cells. Thus, though mating occurs, XX males are not able to sire progeny, presumably because of incomplete germline transformation. In addition, the switch to oogenesis is not enough to allow self-fertilization rendering these worms sterile. As for the other *tra* alleles, *tra(nm1)* X0 worms are not affected. Though a wild type phenotype for *tra(nm2)* males is assumed, X0 worms could not be phenotypically distinguished from XX transformed hermaphrodites. The development of a molecular or genetic marker for males will facilitate the analysis of *nm2* effects on X0 worms.

3.1.3.b) Complementation groups

Crosses using *tra-2(ed23ts)* and *tra-3(ed24ts)* males were used to investigate the allelic relationships between all four *tra* alleles (*ed23*, *ed24*, *nm1* and *nm2*). Progeny were scored for presence of *tra* worms (no complementation) or complete wild type progeny (complementation) (see Material and Methods). Three complementation groups could be identified, corresponding to *tra-2* [*ed23* and *nm1*]; *tra-3* [*ed24*] and *tra(?)* [*nm2*] (*table 5*). Considering that *nm2* has a stronger masculinization phenotype than *ed23* and *ed24*, complements *tra-2* and a putative *tra-3* allele and maps to CIII (Eric Haag, personal communication), a reasonable conclusion is that it represents a *C. briggsae tra-1* allele (see Discussion). Indeed, sequencing of the *C. briggsae tra-1* gene of *tra(nm2)* revealed the presence of a nonsense mutation (Eric Haag, personal communication). Thus, if *ed24* is confirmed as a *tra-3* allele, alleles of all three *C. briggsae* orthologues of the *C. elegans tra* genes will now have been isolated.

3.1.4 Temperature sensitive periods for ed23 phenotypes

To further characterize *tra-2(ed23ts)*, I took advantage of the complete temperature rescue phenotype of worms grown at 16°C to narrow down the essential period when TRA-2 activity is required for normal hermaphrodite development in *C. briggsae*. Given the similarity of phenotypes of *C. elegans* and *C. briggsae tra* mutants, I suspected that these genes function in both organisms at similar times during development. I tested that by comparing the TSP for the mutant phenotypes of *ed23* and a previously described *ts* allele of the *C. elegans tra-2* gene (Klass et al., 1976). I scored for rescue of somatic (tail) and germline (presence of eggs) of animals switched from permissive to restrictive temperatures (shift-up) and from restrictive to permissive temperatures (shift-down) at different times during larval development (*figure 11*).

3.1.4.a) Tail Phenotype

Shift-down experiments indicated that the *tra* tail in *tra-2(ed23ts)* worms started to develop in otherwise wild type looking worms at around 16 hours (all times are adjusted to development at 25°C) post-L1 arrest. 100% of worms shifted to 16°C at 24 hours, corresponding to mid L4 larval stage, developed the incomplete male tail, indicating that wild type TRA-2 activity after this period cannot prevent somatic intersex development. Shift-up experiments mirrored these results. Worms shifted to the restrictive temperature as early as L1 ultimately developed as *tra* worms while shifts after mid L4 did not have an effect and hermaphrodites developed normally. Indeed, the temperature sensitive period (TSP) for *tra* tail development in *tra-2(ed23ts)* worms spanned from mid L3 to mid L4 stages (*figure 18*).

3.1.4.b) Germline phenotype

Rescue of germline masculinization (lack of oocytes) in *tra-2(ed23ts)* hermaphrodites was scored by checking the presence of eggs in the gonad. Though that allowed the identification of gonads that produced both, sperm and oocytes, it did not necessarily mean the complete restoration of wild type fertility levels nor did it take into account gonads that while producing both gametes were unable to self-fertilize due to somatic defects.

The TSP for presence of eggs in shifted *tra-2* worms spanned from late L1 to middle L4 stages. Worms shifted after this time had eggs if grown previously at the permissive temperature (shift-up) or were Tra-like with germ cells committed to make only sperm, if previously grown at the restrictive temperature (shift-down). Differently from the effects observed in the soma, 5% of worms grown at the restrictive temperature and shifted down as early as 9 hours (L1) showed a *tra* germline when adults, even though growth of later larval stages at 16°C ensured normal somatic development (*figure 18*). In fact, TRA-2 activity is needed for correct oocyte production from early L1 throughout larval development.

3.2 Feminizing mutations

3.2.1 Suppressors of tra-2(ed23ts)

Alleles that suppress *tra-2(ed23ts)*-mediated masculinization (*sup* alleles) were isolated in a series of suppression screens. Considering the general organization of the *C. elegans* sex determination pathway in negative regulatory steps, I reasoned that suppression by epistasis of somatic and germline masculization caused by a *tra-2* mutation should be expected of downstream genes that are direct or indirect targets of *tra-2*-mediated feminization in the soma as well as in the germline. In *C. elegans*, the *fem* genes (*fem-1*, *fem-2* and *fem-3*) constitute the major regulatory step for male development, between *tra-2* upstream and *tra-1* downstream in the pathway. Null mutations in all *fem* genes are able to suppress *C. elegans tra-3* alleles (Hodgkin 1986). I therefore hoped to isolate *C. briggsae fem* alleles by selecting for *tra-2(ed23ts)* suppressors though the possibility of also isolating potential *gf* alleles of *tra-1* still existed.

3.2.1.a) Suppressor phenotypes

A total of 800,000 haploid genomes were screened over 8 independent mutagenesis experiments yielding 54 *sup* strains (mutation frequency ~ $6.75e^{-06}$). I specifically isolated worms that showed restoration of self-fertility. Sterile worms, even if suppressed for somatic defects, were not analyzed since these strains could not be immediately maintained. Hermaphrodites and males of *sup* strains were further investigated for somatic and germline phenotypes in order to characterize the level of feminization/suppression involved. Somatic suppression involved the complete restoration of a XX tail, complete vulval development and two-arm gonad. Germline suppression varied from worms with small brood sizes to wild type brood sizes.

3.2.1.a.i- Hermaphrodites

48 (88%) *sup* strains showed complete rescue of the hermaphrodite tail phenotype, 4 (8%) had partial feminization of the tail while 2 (4%) strains showed no alteration from the original *tra* tail phenotype (*figure 19*). Wild type vulva developed in 77% of the strains, 19% had vulva with overgrown tissue (protruded) that did not affect egg laying, and 4% had incompletely formed vulva that prevented egg laying (*egl* phenotype). The germline of all 54 strains were feminized to the extent of allowing production of oocytes, but surprisingly that did not interfere with the early ability to make sperm in L3-L4. In fact, in contrast to *C. elegans fem* mutants, all *tra-2(ed23ts)* suppressor strains isolated were self-fertile hermaphrodites producing both gamete types and maintainable as viable strains at 25°C. Furthermore, I failed in detecting any female *sup* worm that showed the characteristic Fem phenotype observed for alleles of all *C. elegans fem* genes (see Discussion).

Another unexpected phenotype seen at 16°C was embryonic and larval lethality. Though 66% of strains did not show any difference in survival when grown at 16°C and 25°C, 22% and 12% of strains showed some level of embryonic or larval lethality, respectively.

3.2.1.a.ii- Males

Even though the *sup* strains have been continuously cultured for over two years, no typical male or intersex X0 worms were seen in 88% of the strains. Moreover, heat shock experiments, which cause nondisjunction and often results in low frequency of male progeny, did not reveal recognizable males/intersex worms for these same strains. I assumed that in some if not all of these strains, X0 animals are phenotypically transformed into fertile hermaphrodites and become unrecognizable in a population of XX animals. However, since I did not have ways to check the genotypic sex of these worms it is possible that I just failed to identify the male phenotype.

Intersex males with wild type tail but feminized gonad (oocytes in two-arm gonads) were seen in 3 (6%) strains. Though the intersex animals from all three strains had a wild type mating behavior, none were able to sire progeny (*figure 20*). Surprisingly, 3 strains (including DP373) showed wild type males. Though some males had a weak feminization of the tail, they were able to mate and sire progeny at wild type levels.

3.2.1.b) Genetics of suppressor alleles

3.2.1.b.i- Hermaphrodites

Backcrossing *tra-2;sup* hermaphrodites to *tra* X0 males resulted in 100% *tra* XX animals in F1. I concluded that all isolated *sup* strains carried recessive extragenic suppressor alleles and ruled out the presence of intragenic (revertant) alleles. When *tra-2;sup* hermaphrodites were crossed to wild type males, 100% of F1 worms were wild type. Selfed F1 worms from these crosses generated F2 progeny with the classic 3:1 ratio of wild type to *tra* worms. The lack of completely feminized F2 worms supports the conclusion that *sup* alleles do not have a strong effect on the hermaphrodite germline even in a *tra-2+* genotype (*+;sup*). For the 21 *tra-2;sup* strains used for complementation, I checked the F3 progeny of around 15 F2 wild type-looking hermaphrodites in case a maternal effect was present, but never found feminization in this generation either. Presumably these genes do not play a role in hermaphrodite spermatogenesis. Consequently, I resorted to scoring for the ability of the *sup* alleles to suppress *ed23* in double mutants as the sole hermaphrodite phenotype for further genetic analysis.

3.2.1.b.ii- Males

In 15 strains (35%), all F1 heterozygous males derived from either backcrosses (*sup* x *tra*) or outcrosses (*sup* x *wild type*) showed feminization of the germline (strong) and soma (weak). Large oocyte-like cells could be observed inside the gonad of these worms. Attempts to form a vulva and disorganization of tail rays were also observed in some strains (*figure 21*). At first, these results seem to contradict the recessive nature of these alleles in hermaphrodites. Instead, analysis of a null *fem-2* allele suggested that this is a general effect of certain *C. briggsae fem* alleles. Thus, while having a recessive effect on suppression of *tra* hermaphrodite phenotypes, a number of *sup* alleles dominantly feminized X0 worms (see below).

The general genetic characterization of all *sup* strains was followed by the isolation and characterization of one *sup* allele in particular, *fem-B (ed30)* (see below).

3.2.1.c) Complementation of suppressor alleles

I took advantage of the occurrence of fertile males from the *sup* strain DP373 and the availability of a *C. briggsae fem-2* mutant from our collaborator to investigate the number of feminizing genes represented among 21 selected *sup* strains (see Material and Methods).

3.2.1.c.i- *fem-2(nm27)*

A *C. briggsae fem-2* mutant strain (CP36), isolated in a deletion screen in the Haag lab was made available to us. The *nm27* allele is a 1kbp deletion in the 3' end of *fem-2* (*figure 6*) that corresponds to the phosphatase domain of the protein (Pilgrim et al., 1995). The absence of a FEM-2 cross-reaction band Western blots of CP36 lysates (see Appendix A) and the lack of *fem-2* mRNA signal in *in situ* experiments using CP36 worms (Eric Haag, personal communication) suggested that *nm27* is in fact a null allele and no *fem-2* product is present in these worms. *fem-2(nm27)* hermaphrodites, as in the *sup* strains, are fertile worms with no detectable germline feminization defects. Spermatogenesis takes place as attested by the presence of cells that express the sperm-specific marker SPE56 in the adult spermatheca (*figure 6*). The lack of the characteristic stack-of-oocyte phenotype seen in *C. elegans fem-2* females (*figure 6*) and the wild type brood size observed indicated that spermatogenesis is normal (*figure 13*). *fem-2(nm27)* males are fertile hermaphrodites undistinguishable from XX worms (Eric Haag, personal communication). Thus, similar to *tra-2;sup* alleles, *C. briggsae fem-2* appears important for somatic feminization though no effect is observed in the XX germline. I decided to test if there were new *fem-2* alleles among 21 of our *tra-2(ed23ts) sup* strains. I crossed *tra-2 cby-15;fem-2* hermaphrodites with wild type males. F1 X0 worms were somatically wild type males that produced around 20 sperm cells before switching to oogenesis (see below). Young males were able to mate and sire few progeny while males over 1 day after the last molt became sterile. I used these young males in crosses with *sup* hermaphrodites and checked for suppression of the Tra phenotype in F1 XX worms to assess allelic relationships (*figure 8*).

3.2.1.c.ii- DP373 strain

tra-2;sup hermaphrodites of this strain were somatic females with a wild type tail, two-arm gonad and well-formed vulva. However, 30% of XX worms showed a gonad with few germ cells and complete lack of mature oocytes. These sterile worms did not produce progeny even when mated to wild type males, indicating that sperm production was not the cause of sterility. Upon further examination, cells that resembled sperm (with condensed nuclei and granular cytoplasm) were visible outside the spermatheca, in the proximal gonad arms. These cells positively react against a *C. elegans* spermatogenesis-specific antibody (*figure 22*). Thus, in these worms, the germline masculinization of *ed23* is not suppressed by the *sup* allele. Meiosis does not seem to be affected since a normal pachytene zone defined by expression of GLD-1 is present (Jones et al., 1996) (*figure 22*, see Appendix B). However, final oocyte differentiation in the proximal gonad is arrested and cells either abort oogenesis or possibly take a male fate instead (*figure 22*). Supporting this view, the germline of *tra-2;sup* males were not feminized in any way, and these worms were fertile throughout adulthood. I therefore used *tra-2;sup* males in crosses with hermaphrodites of other *sup* strains and checked for suppression of the somatic Tra phenotype in F1 XX worms to establish an allelic group. The gene and its allele responsible for suppression in this strain were named *fem-A* (*ed31)*.

3.2.1.c.iii- Complementation groups

Crosses with 21 *sup* strains with *fem-A* and *fem-2/+* males identified at least three complementation groups: A *fem-2* allelic group (*nm27*, *ed48*, *ed49*, *ed51* and *ed52*), a *fem-A* group (*ed31* and *ed25*), and a group that included *fem-B* (*ed30*) and all other alleles that did not complement either *fem-2* or *fem-A* (*figure 8*). *nm27* and *ed31* were used in the remaining of the genetic analysis as the reference alleles for *fem-2* and *fem-A*, respectively.

3.3.2 Suppressors of tra-3(ed24ts)

3.2.2.a) Suppressor phenotypes

A total of 240,000 haploid genomes were scored over 3 independent suppressor screens yielding 3 *sup* (DP375, 396 and 397) strains (mutation frequency ~ $1.25e^{-05}$). Hermaphrodites and males of *sup* strains were further investigated for somatic and germline phenotypes in order to recognize the level of feminization/suppression.

3.2.2.a.i- Hermaphrodites

Hermaphrodites of all three strains showed complete rescue of the original *tra* masculinization of soma and germline with fertility comparable to wild type levels. No signs of germline feminization were observed.

3.2.2.a.ii- Males

Homozygous males for all three strains were obtained and showed wild type somatic tail and gonad. Males had normal mating behavior and were fertile. In contrast to *tra-2(ed23ts)* suppressors, no signs of germline feminization were ever detected in either sex.

3.2.2.b) Genetics of suppressor alleles

Backcrosses (to *tra* males) and outcrosses (to wild type males) indicated that all three *sup* strains carried autosomal recessive alleles. The presence of fertile homozygous males allowed us to directly test for allelic mutations.

3.2.2.c) Complementation of suppressor alleles

In contrast to *tra-2;sup* strains, homozygous *tra-3;sup* X0 are fertile worms and able to mate. Complementation crosses using *tra-3;fem-C* males indicated that all *sup* strains carried alleles for one gene, named *fem-C*. No *tra* worms were ever seen in the progeny of these crosses suggesting there was only complementation group. I therefore decided to use *ed32* as the reference allele of *fem-C*.

3.2.3 Isolation of fem-B(ed30)

I decided to isolate the *sup* allele in the *tra-2;fem-B sup* strain (DP374) because of two reasons. Firstly, *tra-2;fem-B* hermaphrodites were somatically *tra* worms, with the characteristic snub tail and malformed vulva but fertile due to rescue of the germline defect. This partial rescue phenotype allowed me to distinguish *tra-2;fem-B* worms from *tra-2(ed23ts)* and wild type worms, a feature that other *sup* strains lacked. Secondly, the germline, and to a lesser extent the soma, of *tra-2;fem-B* and *tra-2/+;fem-B/+* F1 males were feminized. These X0 worms were sterile, differently from *fem-2/+* males. Such observations suggested that the *sup* allele (*ed30*) in the *tra-2;fem-B* worms weakly feminizes the soma in males and hermaphrodites while having a stronger effect in the germline of both sexes. Furthermore, complementation results indicated that *fem-B(ed32)* did not group with *fem-2* or *fem-A* (see above), and therefore represented a different suppressor gene.

3.2.3.a) *fem-B(ed30)*

tra-2;fem-B hermaphrodites were crossed to wild type males and the *ed30* allele segregated out of the *tra-2(ed23ts)* background in F2 (see Material and Methods). *fem-B(ed30)* hermaphrodites (DP366 strain) are fertile and show wild type tail and somatic gonad. Brood size analysis failed to show any significant fertility decrease when compared to wild type, suggesting that *fem-B* does not affect XX spermatogenesis (*figure 13*). In contrast, *fem-B(ed30)* males showed feminization of the soma (two-arm gonad, abnormal tail rays) and germline (presence of "ooids") (*figure 20)*. The feminization of *fem-B(ed30)* X0 animals is identical to that observed in *tra-2;fem-B* males. Preliminary data showed that though worms displayed the correct male mating behavior, they were unable to insert the spicules into the hermaphrodite vulva. Whether or not that is the primary cause of sterility has to be further investigated.

The lack of a clear feminization phenotype in *fem-B(ed30)* XX worms suggested the possibility that while feminizing X0 animals, *ed30* might not be the *sup* allele that suppressed *tra-2* in *tra-2;fem-B* worms. Theoretically, *ed30* could represent a secondary mutation carried on from the mutagenesis experiment that was not lost in the *tra-2;fem-B* outcross. To prove that *ed30* was the real *tra-2 sup* allele, I re-introduced it into a *tra-2 cby-15* genotype and checked for the characteristic *tra-2;fem-B*-like phenotypes at 25°C. Such worms (*tra-2 cby-15;fem-B*) were found in the F2 of a cross between *fem-B(ed30)* hermaphrodites and *tra-2 cby-15* males. Backcrossing to *tra-2* males resulted in 100% Tra worms (*tra-2 cby-15 /tra-2 +; fem-B/+*). Thus, *ed30* is a *tra-2(ed23ts)* recessive suppressor allele that restores fertility in *tra-2* hermaphrodites and incompletely feminizes the X0 germline and soma.

3.2.3.b) Inheritance of *fem-B(ed30)* and *fem-2(nm27)*

3.2.3.b.i- Hermaphrodites - Maternal effect

Crosses using *tra-2 cby-15;fem-B* hermaphrodites and *tra-2* males (see Material and Methods) allowed us to verify whether the suppression of the *tra-2* germline masculinization by *ed30* depends on the maternal absence of *fem-B* mRNA or if lack of zygotic expression alone can account for the suppression. Eggs without maternal *fem-B* product but with wild type zygotic expression (m-z+) in the embryo/larva, developed into *tra* worms at 25°C. In contrast, lack of zygotic expression, independent of the maternal contribution (m+z- or m-z-) resulted in *tra-2;fem-B*-like worms (fertile worms with *tra* tail) at 25°C (*table 7*). Therefore the *fem-B* locus shows no maternal effect and zygotic expression of *fem-B* accounts for all feminizing activity of the protein.

Since the *fem* genes in *C. elegans* are known to show strong maternal effect (Hodgkin 1986), I decided to test whether maternal contribution of the *C. briggsae fem-2* mattered in dictating the phenotype of the adult worm. As with *fem-B(ed30)*, I overcame the problem of the lack of an adult phenotype in *fem-2(nm27)* worms by scoring for the ability to completely suppress (soma and germline) *tra-2 (ed23)* XX phenotypes (maternal absence effect). Crosses using *tra-2 cby-15;fem-2* XX worms or *tra-2 cby-15 / tra-2 +; fem-2/+* males (see Material and Methods) clearly showed that worms with maternal contribution and zygotic expression (m+z+) of *fem-2* develop as Tra worms whereas worms lacking both sources of mRNA (m-z-) are wild type hermaphrodites (complete suppression). However, in 47% of worms, the lack of maternal *fem-2* mRNA alone was able to partially suppress the *tra-2* phenotype. m-z+ hermaphrodites were fertile but had the characteristic *tra* tail (*figure 23*), resembling in that respect, *tra-2;fem-B* worms. Therefore, maternal absence of *fem-2* mRNA is sufficient to suppress masculinization of the germline, but not soma, in *tra-2* worms. However, this effect is weak, since lack of zygotic expression of *fem-2* is necessary for complete suppression, as seen in m+z- worms. In conclusion, maternal *fem-2* mRNA plays a minor role in early germline development while later germline and somatic feminization requires zygotic expression. Moreover, zygotic expression of *fem-2* alone is sufficient to ensure all FEM-2 functions in feminizing the worm.

3.2.3.b.ii- Males – Haploinsufficiency

In contrast to hermaphrodites, both *fem-B(ed30)* and *fem-2(nm27)* males showed a distinct feminizing phenotype. *fem-B(ed30)* males are sterile intersex worms, with fairly well formed somatic male structures (*figure 20*) but with germline that produces oocyte-like cells. *fem-2(nm27)* males are completely transformed and fertile hermaphrodites (Eric Haag, personal communication). While checking for maternal effect of these two loci on X0 worms, I noticed that males and hermaphrodites differed in the requirement for wild type gene product during development.

When *fem-B* hermaphrodites were crossed to wild type males, all F1 males (m-z+ X0) had feminized gonads and vulval development that invariably led to sterility (*figure 21*). The degree of feminization observed in the F1 is similar to that of homozygous males (m-z-). Crosses using F1 *fem-B*/+ hermaphrodites and wild type males resulted in 50% feminized males in the cross-progeny despite the presence of both maternal and zygotic expression (m+z+). Thus, a dominant inheritance of *fem-B* mutant phenotypes is seen in males (*table 7*).

Assuming that *ed30* is a loss-of-function allele, one possibility to explain the male-specific feminization of *fem-B*/+ heterozygotes is an X-chromosome location for the *fem-B* locus. I addressed that by using an X-chromosome phenotypic marker, *cby-3 (bd101)*. Segregation of *ed30* was independent of the *cby* phenotype, proving that *fem-B* is not an X-linked gene (see Material and Methods). Since the effect of *ed30* suppression of *tra-2* masculinization in hermaphrodites is recessive (see above), and given the autosomal nature of the allele, I concluded that specifically in males, the wild type product of both alleles of the *fem-B* gene are required to prevent partial feminization of the X0 germline.

None of the *fem* genes in *C. elegans* shows a similar male-specific haploinsufficiency, leading us to ask if other *C. briggsae tra-2 sup* genes also feminize males in the same way as *fem-B(ed30)*. Crosses using either *fem-2(nm27)* hermaphrodites or young *fem-2/+* males revealed that regardless of the maternal *fem-2* mRNA contribution (m-z+, or m+z+), *fem2/+* males were feminized to a degree similar to *fem-B/+* worms (*table 6*). Males were initially fertile, able to mate and sired around 20 cross-progeny. However, 24 hours after emerging from the L4 molt, large oocyte-like cells ("ooids") appeared in the gonads and worms, though able to mate, became sterile (*figure 21*). The feminization of adult *fem2/+* males is restricted to the germline structures and is not as strong as the complete somatic and germline transformation to hermaphrodites seen in a homozygous *fem-2/fem-2* genotype. Therefore, development of correct male soma and germline is dependent of zygotic expression of both wild type alleles of the *C. briggsae fem-2* locus.

3.2.3.c) Suppression of *ed23* by *ed30*

The *fem-B(ed30)* allele suppressed *tra-2(ed23ts)* by epistasis as is evident by the screen from which it was initially isolated. Furthermore, *ed30* is not a temperature sensitive allele, since feminized males also occurred at 16°C. I decided to investigate when during development *ed30* suppressed *ed23*. I assessed that by checking if *ed30* affected or not the previously characterized TSPs for *ed23*. A series of shift-up (16°C - 25°C) experiments with *tra-2;fem-B* worms were performed and hermaphrodite germline analyzed. Presumably, FEM-B directly or indirectly antagonizes TRA-2 activity in the same developmental program that controls germline fate, as attested by the suppressor nature of *ed30*. A logical prediction follows that FEM-B activity is functionally important during the same developmental time as TRA-2. Consequently, the critical period for temperature rescue of the *ed23* germline effects in *tra-2;fem-B* and *tra-2(ed23ts)* animals should be similar, if not identical (*figure 18*), indicating that lack of FEM-B activity was only relevant to suppress *ed23* when TRA-2 activity was required for correct female development. Conversely, the molecular mechanism through which TRA-2 directly or indirectly interacts with FEM-B could determine a change in the time requirement for suppression. In fact, to our surprise, embryos shifted from the permissive to the restrictive temperatures as early as the embryonic comma stage (~550 cells embryo) developed completely wild type germline and soma. Shifts of 2-cell embryos and pre-fertilization shifts resulted in the typical *tra-2;fem-B*-like worms, with *tra* tail and suppressed germline. Therefore, in a *fem-B(ed30)* background, the TSPs for *tra* germline and tail in *tra-2(ed23ts)* worms are pushed back from larval development to late embryogenesis (*figure 24*).

3.3 Genetic interactions

To establish the positions in the *C. briggsae* sex determination pathway for the genes represented by the alleles that have been isolated so far, I analyzed the genetic relationships between alleles by constructing a series of double mutants and checking their somatic and germline phenotypes at 25°C and 16°C (*table 4* and *figure 25*).

3.3.1 tra double mutants

Based on the complementation analysis, three different *tra* genes were present among the masculinizing mutants isolated. Because *nm1* and *ed23* are allelic mutations and display similar phenotypes at 25°C, only *ed23* was used for epistasis analysis of *tra-2*. Two specific phenotypic differences between the *tra* mutants allowed us to discriminate and characterize the epistatic hierarchy of Tra phenotypes: a) *tra-1(nm2)* XX worms are somatically transformed into males and b) the intersex phenotype of *tra-3(ed24ts)*, though very similar to *tra-2(ed23ts)*, is not rescued in all worms grown at 16°C (*figure 13*).

3.3.1.a) *tra-2-tra-3*

tra-2 cby-15;tra-3 is a viable strain at 16°C, reflecting the individual temperature sensitive nature of both alleles. However, as with *tra-3(ed24ts)*, *tra* worms are present at 16°C. At 25°C XX worms develop as *tra* with partially masculinized soma and germline. X0 worms are unaffected fertile males. Though the brood size of the double mutant at 16°C did show a steep decrease if compared to each original *tra* mutant strains, this seemed to be at least partially related to the effect of the *cby-15* allele (*figure 13*).

3.3.1.b) *tra-2-tra-1*

tra-1;tra-2 XX worms displayed the *tra-1* phenotype. XX were transformed sterile males with a completely formed male tail and somatic gonad in both 16°C and 25°C. Males were wild type.

3.3.1.c) *tra-1-tra-3*

tra-1;tra-3 XX worms also showed the *tra-1* phenotype. XX worms were somatically transformed into sterile males at 16°C and 25°C. Males were wild type.

3.3.2 tra-fem double mutants

Epistatic relationships between masculinizing (*tra*) and downstream *sup* genes (*fem-2, fem-B, fem-C)* were characterized based on the double mutant phenotypes (*figure 25*). Of the 6 available *fem-2* alleles (*nm27, ed42, ed48, ed49, ed51* and *ed52*), I used *nm27* as the reference allele because the deletion present in *fem-2(nm27)* most likely results in a null phenotype.

3.3.2.a) *tra-2-fem-2*

tra-2 cby-15;fem-2 XX animals were fertile hermaphrodites at 16°C and 25°C. All somatic and germline masculinization of *tra-2* was completely suppressed by *fem-2*. The brood size was comparable to *cby-15* worms (*figure 13*). Wild type or intersex males were never detected, suggesting males are phenotypically transformed into hermaphrodites as in *fem-2* X0 worms.

3.3.2.b) *tra-3-fem-2*

Similar to the genotype above, the double mutant *tra-3;fem-2* hermaphrodites had completely wild type soma and germline at 16°C and 25°C and brood size comparable to AF16 wild type worms (*figure 13*). Wild type or intersex males were never detected.

3.3.2.c) *tra-3-fem-B*

tra-3;fem-B hermaphrodites, as was the case with *tra-2;fem-B worms*, showed wild type germline though the *tra* tail and masculinization of vulva were not suppressed. Worms were unable to lay eggs at 25°C and died when eggs hatched inside the gonad. At 16°C worms the somatic defects were rescued. Males were feminized as in *fem-B* males.

3.3.2.d) *tra-2-fem-C*

In contrast to the complete germline and somatic suppression of *tra-3* masculinization in the *tra-3;fem-C* hermaphrodites, *tra-2;fem-C* showed no suppression of any *tra-2*-related phenotype. Hermaphrodites were sterile and showed *tra* tail and protruded vulva at 25°C. As expected, *ed23* was completely rescued and the worms were wild type at 16°C. Males were not affected.

4.0 Discussion

4.1 Conserved TRA-2 functions in *C. elegans* and *C. briggsae*

In collaboration with Dr. Eric Haag's lab, I report here the first *C. briggsae* sex-determining mutants, including alleles of the *tra* loci. The presence of two *ts* alleles among the *tra* mutants [*tra-2(ed23ts)* and *tra-3(ed24ts)*] suggests that sex determination in *C. briggsae* is temperature sensitive, a feature also observed in the *C. elegans* pathway. Given the genetic screens performed and the completion of the *C. briggsae* genome project, it seems reasonable to assume at this point that these are the only *tra* genes in the *C. briggsae* sex determination pathway, though the existence of other masculinizing mutations that don't affect dosage compensation cannot be completely discarded. In addition, the hierarchy of TRA proteins in the *C. briggsae* pathway appears to be the same as in *C. elegans*; TRA-2 and TRA-3 likely acting in the same step and upstream from TRA-1.

The analysis of *C. briggsae tra-2* mutants agreed with the knock-down phenotypes obtained in *C. briggsae* RNAi experiments (Kuwabara 1996). *nm1* is a *tra-2* nonsense allele (R1197stop – Eric Haag, personal communication) that predicts a truncated TRA-2 protein lacking most of its intracellular domain, including the predicted TRA-1 and FEM-3 binding domains (*figure 16*). *tra-2(nm1)* hermaphrodites are masculinized to the same extent as *tra-2(ed23)* XX worms grown at 25°C. A similar phenotype is seen for strong alleles of *C. elegans tra-2* (Hodgkin and Brenner 1977). Indeed, *C. briggsae* RNAi against *tra-2*, comparison with *C. elegans tra-2* null phenotypes and lack of *tra-2b*-like transcripts in *C. briggsae* (see below) suggest that *tra-2(ed23ts)* and *tra-2(nm1)* mutant worms could represent the null *tra-2* phenotype in this species. I concluded that, despite poor sequence conservation (*figure 16*), TRA-2 functions in the soma and germline have been conserved in *C. elegans* and *C. briggsae* (Kuwabara 1994, 1996b). Furthermore, the fact that *tra-2* hermaphrodites are not completely transformed into males further suggests that as in *C. elegans* (Goodwin and Ellis 2002), a parallel feminizing signal independent of *tra-2* must exist in *C. briggsae*. This can be clearly seen by the attempt of *tra* worms to form a vulva, indicating the presence of an anchor cell and residual commitment to a female fate

(Kimble and White 1981). Conversely, there is still a possibility that *ed23* and *nm1* are leaky alleles. Analysis of the phenotype of a *her-1-tra-2* RNAi hermaphrodite is needed to confirm whether the *tra-2*-independent feminization is mediated or not by similar upstream regulators of the *C. briggsae* sex determination pathway.

In *C. elegans*, three *tra-2* transcripts exist (Kuwabara et al., 1992). TRA-2A is translated from the largest 4.7kb mRNA transcript that encodes the large transmembrane receptor. TRA-2A has three main domains; the amino extracellular domain (thought to regulate the inhibitory interaction with HER-1, Kuwabara 1996b), the transmembrane hydrophobic and the cytoplasmic domains. TRA-2A activity is necessary to localize the male-determining FEM proteins to the membrane ultimately releasing TRA-1 from repression (Kuwabara 1992, 1995). The cytoplasmic carboxy terminal of TRA-2 can bind FEM-3 (Kuwabara and Kimble 1995; Mehra et al., 1999) and TRA-1 (Wang and Kimble 2001; Lum et al., 200) through different domains. Moreover, both interactions boost the feminizing effect of TRA-2. In addition, adult males express a 1.9kb *tra-2* transcript while the adult hermaphrodite germline expresses a 1.8 kb transcript (Kuwabara et al., 1992). Both transcripts translate a cytoplasmic version of TRA-2 carrying the intracellular domain of TRA-2A but lacking the intermembraneous and extracellular domains coded by the major transcript (Kuwabara et al., 1992). TRA-2B, the product of the adult hermaphrodite transcript, is germline-specific and thought to enhance the feminizing signal by binding to TRA-1 and/or FEM-3 (Lum et al., 2000). Though it cannot completely substitute for TRA-2A activity, TRA-2B has an important feminizing role in the germline and possibly embryo (Lum et al., 2000). Furthermore, a specific role for the cytoplasmic domain of TRA-2A exists in the soma, where its intracellular domain can be released into the cytoplasm through TRA-3-mediated proteolysis (see below) (Sokol and Kuwabara 2000). In *C. elegans* hermaphrodites, maternal *tra-2b* mRNA plays a role in regulating the number of hermaphrodite sperm by feminizing the embryo and early larva (Kuwabara et al., 1998). Because sperm is the limiting gamete in hermaphrodite species of *Caenorhabditis*, the absence of TRA-2b-induced feminization (m-z+) results in increased XX spermatogenesis and larger brood size (Fodor et al., 1983;

Kuwabara et al., 1998). Presumably, the control of spermatogenesis accomplished through maternal *tra-2b* activity is selectively advantageous in preventing the excessive production of sperm in cross-fertilizing hermaphrodites (Hodgkin and Barnes 1991). *C. briggsae* lacks the 1.8kb *tra-2* transcript and all TRA-2 produced derives from the translation of the major 4.7kb mRNA (effectively, TRA-2A). The absence of *tra-2b* mRNA in *C. briggsae* could explain the lack of maternal effect of the *tra-2* locus in this species. In fact, though the *ed23* mutation is not located in the intracellular domain of TRA-2 (see below), no significant difference in the brood size of *tra-2*/+ (m-z+) and wild type hermaphrodites was detected (*figure 13*). Indeed, the transcriptional profiles of *C. elegans* and *C. briggsae tra-2*, predicts a different degree of sensitivity to mutant *tra-2* alleles. In *C. briggsae* the lack of other alternative *tra-2* transcripts encoding proteins with feminizing activity (e.g. TRA-2B) suggests that no compensation to lower TRA-2A activity exists, and therefore XX development should be more sensitive to weak *tra-2* alleles. For this reason, it should not be surprising that a *ts* allele such as *ed23* could cause null-like phenotypes when worms are grown in restrictive conditions.

The *tra-2(ed23ts)* mutation is an A to C transversion in exon 10 of the *C. briggsae tra-2* gene. The predicted mutant protein would have a change of an acidic (aspartic acid) for a neutral (alanine) amino acid at residue 587 in the first extracellular loop of the amino terminal domain of TRA-2 (TRA-2^{D587A}). D587 is conserved both in *C. elegans* and *C. remanei* TRA-2. Considering the location of the mutation, the *lf* and *ts* phenotype in XX worms and the lack of an associated male phenotype, it is not likely that D587A prevents either the docking of TRA-2^{D587A} in the membrane or the binding to HER-1 in males, both roles also assigned to extracellular domains of TRA-2 (Kuwabara 1996a). Though two other *C. elegans tra-2* mutations that disrupt the amino terminal of TRA-2 have been described (Okkema and Kimble 1991), none are mapped to the vicinity of D587, leaving the particular effect of *ed23* in that domain for speculation. Moreover, the primary and secondary structures of the intracellular FEM-3 and TRA-1 binding domains should be intact in TRA-2^{D587A}. Based on the mutant phenotypes at 25°C, I hypothesize that TRA-2^{D587A} in XX cannot assume a correct protein conformation in the membrane, ultimately compromising accessibility of the intracellular binding domains to FEM-3 and/or TRA-1 and consequently affecting the correct flow of the downstream feminizing signal. Since TRA-2 needs to be constitutively on in XX worms to correctly ensure hermaphrodite somatic development, *tra-2(ed23ts)* worms wrongly develop as pseudomales. The folding defect of TRA-2^{D587A} at 25°C may not be as conspicuous at 16°C, allowing restoration of activity in the membrane at this temperature. The effect of TRA-2^{D587A} in the hermaphrodite germline is more complex. Although no clear DRE sequences are found in the 3' UTR of *C. briggsae tra-2* mRNA, a similar functional element must exist since a repressor present in germline lysates binds to it and regulates translation as the GLD-1/FOG-2 complex in *C. elegans* (Jan et al., 1997, 1999). In fact, regulation of *tra-2* mRNA translation in the germline appears to be a general mechanism in *Caenorhabditis* species (Haag and Kimble 2000).

The lack or reduced activity of TRA-2^{D587A} at 25°C bypasses the need for post-transcriptional repression of *tra-2* mRNA and spermatogenesis starts normally in the XX germline. However, because expression of *tra-2(ed23ts)* does not result in TRA-2 activity at 25°C, the switch of germline fate that should result from relaxing *tra-2* mRNA control and repression of *fem-3* mRNA translation (in *C. elegans*, at least) is impaired (Ahringer et al., 1991, 1992). Oogenesis never ensues and the germline is indefinitely committed to a male fate. Consequently, XX *tra-2(ed23ts)* pseudomales showed accumulation of sperm cells in the distal end of the intersex gonad throughout adulthood. These intersex XX *tra-2(ed23ts)* worms never adopted a complete hermaphrodite germline fate (production of oocytes) or somatic male fate (male tail and male behavior). Such phenotypes arise from defects in the commitment to a common sex fate in the germline and soma and eventually result in the adult sterility observed in *tra-2* mutants. It should be noted that the control mechanisms explained above, though present in *C. elegans*, might simply not exist in the *C. briggsae* pathway, in which case an alternative control for the sperm-oocyte germline switch must have evolved in *C. briggsae* (see below).

In *C. elegans* X0 animals, though *tra-2* is transcribed 15 fold less than in hermaphrodites (Kuwabara et al., 1992), TRA-2 activity must be negatively regulated throughout development and adult life to allow formation of male soma and on going sperm production. This is normally accomplished by the male-specific expression of *her-1* (Hunt and Wood 1992). In *C. elegans* males, TRA-2 binds HER-1, an extra-cellular inhibitory factor, preventing the transduction of the downstream signal. Though a rare regulatory mechanism in evolution, cell non-autonomous inhibitory systems are important during development, as with the case of the *patched* receptor in the *Drosophila* Hedgehog signaling cascade (Trent et al., 1991; Perry et al., 1993; Hunter and Wood 1992; Kuwabara et al., 1992). In *tra-2(ed23ts)* X0 worms grown at 25°C, TRA-2^{D587A} is already constitutively off (inactive) and male development independent of HER-1 repression. However, at 16°C, HER-1 becomes necessary again. Either way, TRA-2 activity is repressed and normal male development invariably occurs at both temperatures.

The zygotic expression of *tra-2* during embryogenesis and larval development is essential for correct female fate. Analysis of the critical TSP for the germline and mutant phenotypes of *tra-2(ed23ts)* worms indicated that, as in *C. elegans*, the *C. briggsae* XX germline and soma (tail) have different time requirements when TRA-2 activity is important. Similar to *C. elegans*, TRA-2 activity is needed earlier for the germline than for tail development (Klass et al., 1976). TRA-2 activity has to build up until late L3 when the germline switches from spermatogenesis to oogenesis. On the other hand, male tail morphogenesis requires absence of TRA-2 activity in the soma during middle to late L4. As seen with *ed23*, a faulty TRA-2 protein during most early larval development does not affect normal XX tail development if worms are shifted to the permissive temperature as late as mid L3 stage. Despite these general similarities, the TSPs calculated for progeny production and male tail using the *C. elegans tra-2* allele *b202ts*, are not exactly the same as those for *ed23*. In *tra-2(b202ts)* worms, the TSP for progeny production spans from 10 hours before hatching to 65 hours after hatching while TSP for male tail development starts at 8 hours before hatching and lasts until 8 hours after hatching (Klass et al., 1976). The reason for the differences in both cases most likely relies on differences in scoring phenotypes. Klass et al. scored for rescue to wild type levels of XX fertility in *b202* worms while I considered presence of any number of viable eggs as a sign of restored fertility. Likewise, different degrees of *tra*-induced masculinization of the XX tail were used to classify the somatic defects in *b202* hermaphrodites while the TSP for *ed23* tail accounted for the presence of only two phenotypes; completely rescued worms and worms with any sign of tail masculinization (not rescued).

Finally, genetic epistasis analysis with *tra-1* and *fem-2* indicated that the *tra-2* feminizing role in the *C. briggsae* pathway is accomplished by negatively regulating the activity of at least three *fem* genes. Thus, similar to *C. elegans* (Doniach and Hodgkin 1984; Hodgkin 1986), *C. briggsae* TRA-2 functions as the major feminizing switch in the interface between cells by reading and unifying the sexual development signal in the worm (*figure 25*).

4.2 Lack of maternal effect of *C. briggsae tra-3*

The *C. elegans tra-3* gene encodes a calpain protease involved in cleaving the intracellular domains of TRA-2A (TRA-2ic) (Barnes and Hodgkin 1996; Sokol and Kuwabara 2000). The result cleaved product, TRA-2ic, is a 55KDa peptide that carries roughly the same carboxy region of TRA-2 encoded by the germline-specific *tra-2b* transcript (Sokol and Kuwabara 2000). As TRA-2B, TRA-2ic has intrinsic feminizing activity and plays a minor, though important, maternal role in feminizing oocytes and embryos (Lum et al., 2000).

In contrast to the soma, TRA-3 activity in the embryo and larva has a crucial role in the fate of the *C. elegans* germline. TRA-3 activity during embryogenesis allows the precise timing of germline development by antagonizing translational repression mediated by the binding of LAF-1 to the *tra-2* 3'UTR (see Introduction). TRA-3 cleaves and inactivates LAF-1 to release *tra-2* mRNA for translation (Goodwin et al., 1997). Embryonic TRA-2 activity is thought to prevent the germline from taking an early male fate. The embryonic activity of TRA-3 depends exclusively on maternal *tra-3* mRNA contribution (Goodwin et al., 1997). Not surprisingly, *C. elegans tra-3* shows strong maternal effect (Hodgkin and Brenner 1977). By early larval development, the maternal effect of *tra-3* dissipates, LAF-1-mediated repression of *tra-2* translation ensues, and spermatogenesis starts. Later zygotic *tra-3* expression in L3 worms again shifts the dynamics of LAF-1 repression and TRA-2 levels rise once more to permit oogenesis (Goodwin et al., 1997). The specific control of entry and exit of the germ cell commitment to make sperm is ultimately responsible for the self-fertility potential of hermaphrodites since the number of sperm is the limiting factor for brood size. Though the regulation of *tra-2* mRNA translation in *C. elegans* involves other independent mechanisms (GLD-1-FOG-2), TRA-3 regulation of LAF-1 protein levels is so far the only known feedback mechanism that explains the germline fate switch.

Two aspects of the genetics of *tra-3(ed24ts)* indicate possible functional differences of *C. elegans* and *C. briggsae* TRA-3. Firstly, the degree of somatic masculinization in *tra-3(ed24ts)* is surprisingly stronger than noticed for *tra-2(ed23ts)*. This is clearly seen by the presence of *tra-3* (but never *tra-2*) mutant worms when grown at the permissive temperature. If as in *C. elegans*, *C. briggsae* TRA-3 is a cofactor of TRA-2 and has a secondary feminizing role in the soma, one would expect the phenotype of a *ts tra-2* allele to be stronger or similar to that of a *ts tra-3* allele. Furthermore, the double mutant *tra-2(ed23ts);tra-3(ed24ts)* adopted a clear *tra-3(ed24ts)*-like phenotype (Tra worms at 16°C and reduced fertile progeny), suggesting that *tra-3* is an important player in transducing the TRA-2 signal downstream in the soma (*figure 13*). Since the molecular lesion for *ed24* is not yet known, there is still a possibility that *tra-3(ed24ts)* masculinization represents the null phenotype while the *tra-2(ed23ts)* phenotype does not. Alternatively, differences in the type of mutation involved in *ed23* and *ed24* could also explain the differences in degree of masculinization in these mutants. Secondly, in contrast to *C. elegans tra-3* (Hodgkin and Brenner 1977), *C. briggsae tra-3* shows no maternal rescue. *tra-3/tra-3* worms from *tra-3/+* mothers are masculinized at the restrictive temperature to the same extent as m-z- XX animals. That brings up the question of whether translational regulation of *tra-2* in *C. briggsae* is mediated by LAF-1 and if so, how the timing for start of spermatogenesis is controlled in the XX germline. The question becomes even more crucial considering that in *C. briggsae*, the GLD-1-FOG-2 control of *tra-2* translation in the hermaphrodite germline is absent and consequently the mechanism through which hermaphroditism is established is unknown (Nayak et al., 2004).

Interestingly, the lack of a *tra-2b*-like transcript and the absence of maternal TRA-3 indicate that in *C. briggsae* oocytes, the intracellular domain of TRA-2 is never produced in a soluble form and therefore should be dispensable for normal hermaphrodite development in this species. Though there is no data at the moment on whether or not *C. briggsae* TRA-3 cleaves TRA-2 to generate a TRA-2ic-like peptide, it is possible that this secondary feminizing route evolved specifically in *C. elegans* after the split from the *C. briggsae* common ancestor. A similar event that resulted in the evolution of FOG-2 from an ancestral F-box gene occurred specifically within the *C. elegans* lineage (Nayak et al., 2004).

Epistasis analysis of *tra-3* and the other available sex-determining alleles indicates that *C. briggsae tra-3* occupies a similar position in the pathway as its *C. elegans* homologue (Hodgkin 1996). *C. briggsae tra-3* is upstream from *tra-1*, *fem-2* (soma/germline) and *fem-B(ed30)* (germline) and possibly occupying the same molecular niche in the pathway as *tra-2*.

The differences in the mechanisms of germline feminization and the apparent strong effect in the soma, suggests that, as *fem-2* (see below) and *gld-1* (Nayak et al., 2004), *tra-3* functions could have significantly changed in the *C. briggsae* sex determination pathway.

4.3 TRA-1 converges the feminizing signals of TRA-2 and TRA-3

Contrasting to *tra-2* and *tra-3* mutants, the soma of *tra-1(nm2)* XX worms is completely transformed into a male phenotype. The somatic masculinization observed in *C. elegans tra-1* null mutants (Hodgkin and Brenner 1977) can be rescued by the expression of *C. briggsae* wild type *tra-1*. However, in these inter-specific experiments, the somatic gonad and germline are never rescued (de Bono and Hodgkin 1996). Supporting the interpretation that *nm2* is null allele, a nonsense mutation (Q512stop) is found in the *tra-1* gene of *tra-1(nm2)* animals, suggesting that TRA-1 activity could be largely abolished in these worms (Eric Haag, personal communication). Furthermore *C. briggsae tra-1* is epistatic to *tra-2* and *tra-3* genes and occupies the most downstream position in the sex determination pathway among the cell-autonomous genes with a feminizing role identified so far (*figure 25*).

However, the germline of *tra-1(nm2)* XX worms does not completely adopt a male fate as some *C. elegans tra-1* null mutants do. While *tra-1* XX males in *C. elegans* can be fertile (Hodgkin 1987), the lack of *C. briggsae* TRA-1 activity in *tra-1(nm2)* XX worms is not enough to completely erase the hermaphrodite germline program in the adult. Though transformed XX worms produce sperm and engage in mating behavior, they are sterile. Furthermore the germline eventually shifts to produce oocyte-like cells ("ooids"), indicating that some XX identity remains, a feature also seen in some *C. elegans tra-1* alleles (Hodgkin 1987). These "ooids" are not viable gametes and self-fertilization does not occur either. Whether sterility is due to a germline identity problem or a specific somatic defect remains to be investigated. Determining whether *nm2* is or not a null allele will clarify if the *C. briggsae* germline has a TRA-1-independent feminizing activity.

Another unanswered question concerns the specific role of *tra-1* in the *C. briggsae* spermatogenesis in males and hermaphrodites. In the *C. elegans* germline the *fem* genes, and not *tra-1*, are the final regulators of sexual fate. Furthermore, though *tra-1 gf* mutations completely feminize XX worms, *C. elegans* hermaphrodites with *tra-1 lf* alleles actually make less sperm. Presumably, while having a major feminizing role, *tra-1* also positively regulates spermatogenesis (Schedl et al., 1989). It will be important to analyze the somatic and germline phenotype of *tra-1-fem-2* double mutants, in particular the effects on XX spermatogenesis, to understand if the conservation of TRA-1 functions in the soma is also extended to the germline in *C. briggsae*.

In conclusion, the transformed phenotype of *tra-1(nm2)* XX worms, its genetic interactions with *tra-2* and *tra-3* alleles and the previously described RNAi phenotype (de Bonno and Hodgkin 1996) are all consistent with a role of *C. briggsae* TRA-1 as the final regulator of sexual fate in the soma.

4.4 At least three *C. briggsae fem* genes

Our hunt for suppressors of *C. briggsae tra-2(ed23ts)* yielded 54 *sup* alleles. These alleles feminize the original *tra-2* worm in the sense that they allow the normal development of the hermaphrodite soma and the sperm-oocyte switch in the germline. The original *C. elegans* saturation screen for the *fem* genes was performed by isolating suppressors of a weak *tra-3* allele (Hodgkin 1986). 26 *lf* alleles of three genes (*fem-1*, *fem-2* and *fem-3*) were isolated in addition to 14 *gf tra* mutations (Hodgkin 1986). Similar to the alleles isolated in our suppression screens, the *C. elegans tra-3 sup* alleles restored normal female development of somatic structures. Contrary to expectations, the feminizing effects of these alleles in the germline appear to be different. The most characteristic phenotype of strong alleles of all three *C. elegans fem* genes is the transformation of hermaphrodites into fertile females due to complete feminization of the XX germline (Nelson et al., 1978; Kimble et al., 1984; Hodgkin 1986). Consequently, *C. elegans* hermaphrodites with null *fem-2* mutations only produce oocytes and cannot be maintained as homozygous stocks. In addition, X0 males are completely transformed into fertile hermaphrodites only when no maternal or zygotic *fem* expression is present, whereas m-z+ males are intersex animals due to the absence of maternal *fem-2*. Thus, in *C. elegans*, *tra-3* and *tra-2* suppressors characterize a specific group of feminizing genes (*fem*) whose activity is essential for all aspects of male development and transient hermaphrodite spermatogenesis (Hodgkin 1986).

Surprisingly, I have not been able to isolate *tra-2;sup* alleles that completely feminize the hermaphrodite germline. On the contrary, the *sup* strains show different degrees of somatic suppression but are otherwise fertile, producing first sperm and later oocytes even though some strains show a distinct reduction in fertility. Despite the absence of the classic feminizing phenotype, I have named our alleles as members of *C. briggsae fem* genes due to their conserved position in the pathway (downstream of *tra-2*). Complementation analysis shows that at least three different *fem* genes are important for the *C. briggsae* sex determination pathway, one being the *C. elegans fem-2* orthologue. Given that only 21 *tra-2 sup* strains were tested, and that the members of the *non-fem-2;non-fem-A* group have not been tested for non-allelic mutations, I cannot discard the possibility that there may be more than three *fem* loci in *C. briggsae*. Furthermore, as in *C. elegans* (Hodgkin 1986), *C. briggsae fem-2* shows maternal effect (though weak) in suppressing *tra-2* masculinization in double mutants. The fact that m+z- double mutant worms (*tra-2;fem-2*) were somatically *tra* but otherwise fertile, indicates that maternal *fem-2+* normally plays a role in the germline (inhibiting entry in oogenesis) while zygotic *fem-2+* is needed for correct somatic development in addition to its germline role (*table 6*).

I isolated the *sup* allele *(ed30)* from one *sup* strain (DP374). *ed30* is not a *fem-2* allele nor does it complement the mutations in *fem-A*, the third complementation group. *ed30* has no effect on hermaphrodite spermatogenesis but feminizes the male germline (*figures 13 ,20*). In that respect, *ed30* behaves as a *C. elegans fog* allele. The *C. elegans fog-1* and *fog-3* are germline-specific genes acting downstream of *tra-2*. As *ed30*, the *C. elegans fog-1* and *fog-3 lf* alleles suppress germline masculinization of *tra-2* mutants without affecting the soma of XX worms. However, mutations in the *C. elegans fog* genes also transform hermaphrodites into fertile females (*fem*-like phenotype). Though *fem-B(ed30)* also has little or no somatic effect on the *tra-2* masculinization phenotypes, its germline effect does not completely feminize the hermaphrodite either, since worms remain self-fertile. In fact, resembling the *C. briggsae fem-2* mutant phenotype, *ed30* apparently can restore oogenesis but plays no major role in regulating spermatogenesis. If *fem-B* is in fact the *C. briggsae* orthologue of *fog-1* or *fog-3*, (i) *ed30* is a very weak allele or (ii) spermatogenesis in *C. briggsae* depends on a completely different set of genetic elements than those used by the *C. elegans* germline or (iii) the molecular orthologues have different functions. Unexpectedly, genetic interaction data suggest that temperature rescue of the *ed23* somatic and germline Tra phenotypes happens earlier in development in *tra-2;fem-B* worms than the timeframe defined by the tail-TSP in *tra-2(ed23ts)* hermaphrodites (*figure 24*). Presumably, the (direct/indirect) inhibition of FEM-B by TRA-2 activity takes place in early embryogenesis though the effect on morphogenesis is only seen in later developmental events in the hermaphrodite. The ability of *ed30* to affect the requirement of TRA-2 during development and the weak, though present, *mab* (male abnormal)-like abnormalities seen in the soma of *fem-B* males (*figure 20*) advocates against *fem-B* being an orthologue of either *fog-1* or *fog-3*. In addition, the RNAi phenotype of *fog-3* suggests a feminizing activity of this gene in *C. briggsae* (Chen et al., 2001, see below). Assuming that in *C. briggsae* the *fog* genes have a germline-specific role, as apparent by their RNAi phenotypes, somatic effects such as those noticed in *fem-B(ed30)* worms should not be expected. Yet again, given the clear lack of functional conservation of the *fem* genes demonstrated here, there is also a possibility that *C. briggsae fog-1* and *fog-3* have a broader role in sex determination.

fem-A(ed31) successfully suppresses the soma and germline effects of *tra-2* without causing strong feminization in males. Even in the small number of males that do show a slightly abnormal ray arrangement in the tail, the germline is never affected. In fact, as in the case of the *tra-3* suppressor *fem-C(ed32)*, *ed31* feminizes the soma and restores oogenesis in hermaphrodites without significantly affecting either tissue in males. The lack of a male phenotype is rare among other suppressors and clearly distinguishes *fem-A* from *fem-B* and *fem-2* alleles. Since the molecular lesion in *tra-2(ed23ts)* is not an amber mutation (*figure 15*), the suppression seen in *tra-2;fem-A* hermaphrodites derives from a true epistatic relationship indicating that *fem-A* is a sex-determining gene. Furthermore, given the strong suppression seen in the XX soma, it is unlikely that *ed31* is a weak allele. To date, there are no known genes in the *C. elegans* somatic or germline sex determination pathways whose null allele suppresses *tra-2* without causing feminization of the male germline. In fact, null alleles of *fem-1*, *fem-2*, *fem-3*, *fog-1* and *fog-3* equally transform the male germline. Interestingly, though most *tra-2;fem-A* hermaphrodites are fertile, few show incomplete suppression of the germline masculinization phenotype. The germ cells of these worms correctly enter meiosis and progress through prophase I but oocytes fail to develop and apparently adopt a male fate instead (*figure 22*). The low penetrance of male somatic feminization and lack of suppression of germline Tra phenotypes in the hermaphrodite are puzzling. The isolation of the *fem-A* allele in a *tra-2+* background and epistasis with *tra-3* and *tra-1* should help to better understand the role of FEM-A in sex determination.

In a way, *ed31* and *ed30*, both suppressor alleles of *tra-2(ed23ts)*, have different impacts on sex determination in *C. briggsae*. While *ed30* weakly feminizes the XX germline but has no significant effect in the soma, *ed31* has mostly no effect in the male germline, but completely suppresses the somatic structures in *tra-2* worms. Indeed, the feminizing activity of *ed30* is mostly manifested in the germline while *ed31* primarily affects the soma. Further molecular identification of *fem-B* and *fem-A* will be required to fill the gaps in the differences of somatic and germline sex determination in *C. briggsae*.

4.5 Protein requirements for development of the *C. briggsae* male

The X0 feminization noticed in *fem-B(ed30)* males occurs in a dominant fashion, even though the suppression phenotype in hermaphrodite is clearly recessive. *fem-B/+* males from heterozygous parents show the same degree of germline feminization as that of the homozygous mutants (*table 7*). Moreover this effect is not maternal, since feminization also occurred in the *fem-B/+* male progeny from *fem-B/+* mothers. Surprisingly, this sex-specific haploinsufficient effect is seen in a significant number of isolated *sup* strains (*figure 8*), including *fem-2* males, though in this case homozygous *fem-2* X0 worms are completely transformed while heterozygotes are intersex (*table 6)*. Young *fem-2/+* males produce few sperm cells and can sire progeny when crossed, but later in life become sterile when the germline shifts to oocyte production. As seen before, this contrasts with the phenotypes of *tra-2;fem-A* males and its allelic strain, DP378, whose males have wild type germlines. Though maternal *fem* mRNA is essential for complete X0 development in *C. elegans*, feminization is never seen in a *fem-2/+* genotype with wild type maternally contributed *fem-2*. In fact, *C. elegans* X0 development is not sensitive to changes in zygotic *fem-2* dosage (*table 6*) as seen by the recessive nature of these alleles in both XX and X0 worms (Hodgkin 1986). Curiously, *fem-3* in *C. elegans* seems to be needed in two doses for full fertility in XX animals (Hodgkin 1986), indicating a dominant effect in the XX spermatogenesis. However, none of the *C. elegans fem* genes, including *fem-3*, are haploinsufficient for male spermatogenesis. In fact, though zygotic *fem-3* expression is not sufficient for complete male development in *C. elegans*, *fem-3/+* (m+z+) X0 worms are completely normal (Hodgkin 1986). I reasoned that in *C. briggsae*, but not *C. elegans*, expression of two copies of *fem-2*, *fem-B* and possibly other *tra-2 sup* genes (but not *fem-A*) is needed to maintain spermatogenesis in the male throughout adulthood. The absence of one functional allele, even when maternal contribution is supplied (e.g. m+z+ heterozygotes), ultimately results in germline feminization.

In *C. briggsae*, the differences of *fem-2* requirement for XX spermatogenesis (where it is dispensable) and X0 spermatogenesis (where it is haploinsufficient), underlies the variability of molecular mechanisms that evolved in the germline of different sexes to arrive at a common result. It further supports the idea that sex determination pathways in *Caenorhabditis* are flexible enough to support functional adaptations in a tissue-specific level. Furthermore, why the male germline needs a higher dose of FEM-2 than the soma becomes an important issue. In *C. elegans* males, both somatic and germline fates depend on continuous HER-1 repression of TRA-2 activity (Schedin et al., 1994). In *C. briggsae fem-2/+* males, a haploid dose of *fem-2* is enough to repress TRA-1 in the soma and allow formation of male structures. However, in the germline, while a single *fem-2* dose can trigger the onset of spermatogenesis, it is somehow unable to prevent the shift of germline towards oogenesis in the adult. How can the male germline adopt a female fate without freeing TRA-2 from HER-1 repression? Before addressing this question, the role of HER-1 in *C. briggsae* males needs to be clarified. *C. briggsae* HER-1 can masculinize hermaphrodites, as seen in XX worms carrying *her-1* transgenes (Streit et al., 1999). However, it is not known if *her-1* has the same masculinizing activity in the male germline as its *C. elegans* orthologue does. The isolation of *C. briggsae her-1* alleles or the introduction of *her-1* transgenes will be needed to confirm that.

The unexpected dominant effect of *tra-2 sup* alleles in the commitment of the male germline to spermatogenesis adds some interesting new information on how spermatogenesis may be regulated in *C. briggsae*. In principle, the male germline is potentially able to switch fates toward oogenesis even after the decision to undergo sperm production has been made. In that respect, it is similar to the hermaphrodite germline. The fact that the product of the *fem* genes is needed in specific amounts for male development further indicates that, as in the soma, these proteins have a downstream feminizing target which is the final regulator of germline fate. A failure to inactivate this factor drives the germline to a female fate. In a "*fem-x/+*" male, for example, not enough "FEM-X" activity would exist to repress the final downstream regulator, which would ultimately lead to an oocyte-producing germline. At this point it is inevitable to consider TRA-1 as the probable target of the FEM proteins in the germline as well as in the soma. It should be noted that the mechanism through which the *fem* genes in collaboration with *fog-1* and *fog-3*, determine male fate in the *C. elegans* XX and X0 germline is not well understood. Furthermore, though these genes are epistatic to *tra-1* in the germline, none encodes a protein with an apparent role in transcriptional regulation. Therefore, it is still possible that a yet unidentified downstream feminizing gene regulates germline sex determination in *C. elegans* and *C. briggsae*.

Could sex determination in the *C. briggsae* soma and germline rely on repression/activation of TRA-1 as the final regulator in the pathway? The answer to this question lies in solving the exact role of *C. briggsae tra-1* and will have deep consequences in understanding the evolutionary history of the *Caenorhabditis* sex determination pathways.

4.6 *C. briggsae fem-2* does not control the onset of XX spermatogenesis

Aside from the general low sequence conservation between sex-determining orthologues, the primary finding that prompted further research into possible structural differences in the *C. briggsae* and *C. elegans sex* determination pathways involved the lack of germline phenotype in *C. briggsae fem-2* RNAi worms (Hansen and Pilgrim 1998; Stothard et al., 2002). Recently, *fem-1* (Jeb Gaudet, personal communication) and *fem-3* (Eric Haag, personal communication) RNAi experiments in *C. briggsae* provided similar results. Since RNAi does not work as well in the germline, a possibility existed that the lack of germline feminization derived from a technique limitation. However, the normal fertility of *C. briggsae fem-2 (nm27)* hermaphrodites proves that *fem-2* does not in fact play a significant functional role in hermaphrodite spermatogenesis (*figure 6*). Furthermore, hermaphrodites of five other *C. briggsae sup* strains with *fem-2* mutations (*ed42, ed48, ed49, ed51 and ed52*) are also fertile. Given that no *fem-2* mRNA signal is detected in *in situ* hybridizations of *fem-2(nm27)* hermaphrodites (Eric Haag, personal communication) and FEM-2 is apparently absent from CP36 lysates (see Appendix A), *nm27* is likely a null *fem-2* allele. In addition, the lack of a true Fem phenotype in the five *C. briggsae fem-2* mutant strains isolated in this work is consistant with a functional difference for this gene in *C. briggsae* and not the result of hypomorphic alleles. These observations support the interpretation that the lack of hermaphrodite germline phenotype for *C. briggsae fem-1* and *fem-3* RNAi are indeed meaningful. A reasonable conclusion is that sex determination in the hermaphrodite germline occurs independently of the FEM complex. However, since *fem-2* alleles suppress the masculinization of *tra-2* in the XX germline by restoring oogenesis (see above), this gene, and likely the other *fem* genes, must also have a distinct role in germline sex determination. For example, it is possible that in *C. briggsae* the control of germline fate is split and different regulatory steps exist to regulate spermatogenesis and oogenesis (*figure 26*).

The ability of the XX germline of *tra-2;fem-2* and *tra-3;fem-2* double mutants to switch back to oogenesis at the correct developmental time indicates that *C. briggsae fem-2* is responsible for buffering the female signal coming from the *tra* genes upstream (*figure 25*). Presumably this is accomplished by the conservation of the negative interaction with TRA-2 and FEM-3 (Kuwabara et al., 1992) in the germline (Paul Stothard, personal communication). However, the downstream effects of *fem-2* do not extend as far as to regulate the onset of spermatogenesis. One explanation for that involves the redundancy of FEM-2 masculinizing activity with one or more of the other final regulators of germline sex determination. Accordingly, the germline of *fog-3* RNAi worms in *C. briggsae*, contrary to what is observed with the *fem* genes, is feminized (Chen et al., 2001). Therefore, FOG-3 activity could be independent of FEM-2 and sufficient to induce spermatogenesis in the germline of hermaphrodites. Moreover, the direct binding of TRA-2ic to TRA-1 has been implicated with the onset of hermaphrodite spermatogenesis in *C. elegans* (Wang and Kimble 2001). Indeed, if that interaction proves true in *C. briggsae*, the germline fate could rely mostly on the activity of the TRA proteins. Since not much is known about how *fem-1, fem-2, fem-3, fog-1* and *fog-3* regulate the germline fate, it is hard to dissect what specific molecular changes must have happened in the *C. briggsae* pathway to bypass the need of FEM-2 activity seen in *C. elegans*. Alternatively, the *fem-2* masculinizing role might have evolved specifically in the germline of *C. elegans*, from an ancestral *fem-2* gene that lacked activity in this tissue. In that case, the function of *C. briggsae fem-2* might resemble its original role in the ancestral pathway. Moreover, it is noteworthy that removing FEM-2 activity results in a small but significant decrease in brood size of selfed hermaphrodites, presumably due to a reduction in the number of sperm cells produced in the XX germline (*figure 13*). Interestingly, this effect is amplified when *tra-2*, *tra-3* or both are knocked out in a *fem-2(nm27)* background, though this is harder to interpret in the case of double mutants with the *tra-2 cby-15* chromosome, due to the lower fertility characteristic of the *cby-15* allele (*figure 13*). Therefore, it still possible that *C. briggsae fem-2* plays a secondary role in maintaining spermatogenesis.

The feminization observed in the germline and soma of *C. elegans fem-2* X0 animals (Kimble et al., 1984) is conserved in *C. briggsae fem-2(nm27)* males. These worms are fertile, wild type-looking hermaphrodites (Eric Haag, personal communication). *C. briggsae fem-2* is wholly epistatic to both *tra-2*, *tra-3* and to *fem-B(ed30)* in the germline. Furthermore, *C. briggsae fem-2*, but not *fem-B*, is maternally inherited as seen by the germline rescue from *tra-2* masculinization in *fem-2/+* (m-z+) worms. Maternal contribution of *fem-2* thus plays a role in masculinization, but the same does not happen with *fem-B* whose effect in X0 development can be completely accomplished through expression of the zygote's genome alone. As is the case with *C. elegans*, complete suppression of Tra phenotypes in the double mutant (*tra-2;fem-2)* is dependent on the lack of both maternal and zygotic *fem-2* expression (Hodgkin 1986). Thus, the maternal inheritance, epistatic relationships and role of *C. briggsae fem-2* in the X0 germline and soma of XX and X0 worms are similar to *C. elegans fem-2* (Hodgkin 1986).

4.7 *tra-3* suppressors may be amber alleles

All three *tra-3(ed24ts) sup* alleles isolated are *lf* mutations that represent one single complementation group. The fact that *fem-C(ed32)* cannot suppress *tra-2(ed23ts)* though *tra-3(ed24)* is suppressed by *tra-2(ed23ts) sup* alleles (e.g. *ed30* and *nm27*) in addition to the lack of feminizing phenotypes in X0 animals suggest two possibilities. First, *ed24* could represent a nonsense *tra-3* mutation and *ed32* an amber suppressor. Since *ed23* is not an amber allele (see above), it should only be suppressed by epistasis, explaining the lack of suppression of *tra-2* by *ed32*. Furthermore, sex-determining phenotypes would not be expected of an informative suppressor. The second explanation would have to assume the existence of a new masculinizing gene in *C. briggsae*, yet unknown in *C. elegans*, downstream of *tra-3* and upstream or in parallel to *tra-2*. The new gene would be a target of TRA-3 but not TRA-2 and form a new masculinizing step in pathway. Hence, the first possibility is more likely. I therefore speculate that *fem-C* is in fact not a gene with a role in sex determination, but rather, encodes a mutant $_t$RNA. Testing this hypothesis will require the sequencing of the mutation in *tra-3(ed24ts)* and the isolation of other *C. briggsae* amber alleles to test suppression in genetic crosses.

4.8 Rapid sequence divergence and conservation of protein function

To understand how the sex determination pathways in *Caernorhabditis* evolved, the significance of the striking sequence divergence between sex-determining genes of different species will have to be ultimately explained.

Strong evidence has mounted to make the case that the *fem* genes, and in particular *fem-2*, are evolving faster than other non-sex-determining genes. The *fem-2* genes in *C. elegans*, *C. briggsae*, *C. remanei* and a fourth *Caenorhabditis* species (CB5161) have diverged far more than the other PP2C members in these species (Stothard et al., 2002). The isolation and analysis of *C. briggsae fem-2* alleles in this study proves that FEM-2 evolution in the *C. briggsae* lineage either resulted in loss of germline function or conservation of a primitive role in the pathway that did not involve controlling XX spermatogenesis. If we accept the relevance of *C. briggsae* RNAi experiments for *fem-1* and *fem-3* genes, the whole FEM complex in *C. briggsae* does not affect XX spermatogenesis. Given that the majority of the remaining identity between the FEM-2 proteins is concentrated at the phosphatase domain in the carboxy terminal (Stothard et al., 2002), and that FEM-2-mediated phosphatase activity is necessary, though not sufficient, for its role in sex determination (Hansen and Pilgrim 1998), we speculate that changes in the amino terminal domain of these enzymes are responsible for the apparent germline loss-of-function of the *C. briggsae* or gain-of-function of the *C. elegans* FEM-2 proteins. Indeed, at least for *fem-2* and possibly for the other *fem* genes, rapid sequence divergence clearly underlies changes in protein function. Because the fate of the XX germline is particularly susceptible to these changes, further studies of pathway evolution and speciation using this model should concentrate on the molecular mechanisms that control hermaphroditism in *Caenorhabditis*.

The similarity of *C. elegans* and *C. briggsae tra-1*, *tra-2* and *tra-3* mutant phenotypes contrast with what is observed for *fem-2*. Though the *tra* genes have evolutionarily diverged even more than *fem-2* at the protein level in *Caenorhabditis* (Stothard and Pilgrim 2003), their functions apparently have not changed. Conservation of the TRA-1 role in sex determination extends beyond the *Caenorhabditis* genus (Pires-daSilva and Sommer 2004). In fact, rapid sequence divergence does not always correlate with functional changes. The co-evolution of members of a pathway in a species could explain the apparent contradiction of growing sequence divergence between orthologues and the conservation of function observed (Stothard and Pilgrim 2003; Haag and Ackerman 2005). Such patterns of controlled evolutionary "drift" are widespread in the history of developmental pathways and may indeed be the rule instead of the exception (True and Haag 2001). In other words, pathway structure and its role can be conserved even though its orthologues in different species are no longer functionally interchangeable. Substantiating this theory, the protein interactions of TRA-1 and TRA-2, TRA-2 and

FEM-3 and FEM-2 and FEM-3 are mostly species specific (Lum et al., 2000; Haag et al., 2003 and Paul Stothard, personal communication). An interesting exception is *C. briggsae* FEM-3, which binds *C. briggsae* FEM-2 and also the *C. elegans* and *C. remanei* orthologues promiscuously (Paul Stothard, personal communication).

4.9 Dynamics of pathway building

Sex determination in *Caenorhabditis* is governed by a highly divergent Hedgehog-like pathway built on a succession of intercalated inhibitory steps that define a hierarchy of function. If co-evolution conserves the overall output of the pathway by shaping individual protein-protein interactions in different species, how does the pathway lose or gain new factors? The *C. briggsae* FEM complex has lost (or gained) its ability to induce spermatogenesis in the XX germline, though it still does that in the male germline. Moreover, its downstream control of TRA-1 in the soma and upstream interaction with TRA-2 are still essential for correct male fate in all tissues. Although there are no candidates to date for proteins that interact with FEM-2 in the germline, changes in the affinity for its binding partners could account for the different roles in the *C. elegans* and *C. briggsae* pathways. Supposing that the original binding partner of FEM-2 in an ancestral *Caenorhabditis* species shared conserved domains with members of a protein family, co-evolution of the binding domain that mediates the interaction with FEM-2 could eventually add other members of the same protein family as potential FEM-2 targets (Stothard and Pilgrim 2003). Indeed *fog-1* and *fog-3*, both thought to regulate the ultimate oocyte/sperm fate decision of the XX and X0 germline, are members of large protein families (Chen et al., 2001). FOG-1 is a cytoplasmic polyadenylation element binding protein (Barton and Kimble 1990; Jin et al., 2001; Luitgens et al., 2000) and FOG-3 is a member of the Tob protein family (Ellis and Kimble 1995; Chen et al., 2000). Eventually, the increase in binding partners could create functional redundancy of the FEM-2 mediated step, consequently decreasing its functional importance in the pathway. Indeed, recruitment of proteins from one pathway into another seems to be a dominant force in the evolution of metabolic pathways (Teichmann et al., 2001; Copley and Bork 2000). Without evolutionary constraint to maintain the integrity of the binding domains, FEM-2 would eventually exit the pathway or form new parallel signaling routes with multiple binding partners, as seen in the TRA-2 binding of both FEM-3 and TRA-1 (Goodwin and Ellis 2002).

The entry and exit of members in the sex determination pathway during evolution predicts the existence of genes that while belonging to the pathway, remained with, or acquired new, non-sex-determining functions. Evidence for this transitional state has recently been discovered in *C. elegans*. Independently from its role in the sex determination pathway, *C. elegans* TRA-1 is important for development of the somatic gonad in males and hermaphrodites alike. TRA-1 controls the maturation of the somatic gonadal precursor cells (GPC) and their correct divisions in L1 larvae (Mathies et al., 2004). Moreover, FEM-2 is involved in the elongation of the *C. elegans* embryo by controlling actin/myosin contractions in embryonic cells much earlier in development than its function in somatic and germline sexual decisions (Piekny et al., 2000). Interestingly, both TRA-1 and FEM-2 non-sex determining functions appear to be redundant with other proteins. The C2H2 zinc-finger protein EHN-3 also promotes correct somatic gonad development by controlling the GPC fate. In fact, whereas single mutants for *tra-1* and *ehn-3* have mostly normal somatic gonadal development, GPC in *tra-1-ehn-3* double mutants do not mature or divide (Mathies et al., 2004). Similarly, the embryonic role of FEM-2 is redundant with the major elongation pathway mediated by the Rho-binding kinase LET-502 and its antagonist, the myosin phosphatase MEL-11 (Piekny et al., 2000). Based on the conservation of the sex-determining functions, it is possible that FEM-2 activity during embryogenesis and TRA-1 activity in early larval development are the ancestral (FEM-2) or derived (TRA-1) roles of these proteins evolving independently of their sex determination roles.

Examples of how the sex determination pathway builds connections is supported by the discovery of specific control loops between members of the pathway that, at first, seem to contradict the mainstream role of these proteins in the cascade. For instance, *C. elegans* TRA-1, in addition to controlling transcription of female-specific genes and repressing expression of male-specific genes in XX animals, has a critical role in the export of *tra-2* mRNA out of the nucleus. TRA-1 binds to a 3'UTR region of *tra-2* mRNA named TRE (*tra-2* nuclear retention element) which overlaps, but is not exactly the same, as the one necessary for translational repression in the germline of hermaphrodites (DRE, see introduction) (Graves et al., 1999). Presumably, TRA-1 directs the export of *tra-2* mRNA through an LMB-sensitive export pathway by competing with nuclear retention factors for binding to TRE. The removal of TRE or specific *trans*-factors of the *C. elegans* mRNA export machinery (CeNXF-1, CeREF-1, CeREF-2) results in the incorrect adoption of the NXF-1 pathway for mRNA transport. The switch of export machinery used in shipping mRNA out of the nucleus affects translation of *tra-2* mRNA in the cytoplasm (Kuersten et al., 2004). Therefore, a TRA-1/*tra-2* mRNA complex is necessary for TRA-2 activity as much as TRA-2 repression of the FEM complex is essential to activate TRA-1. However, the export of TRA-1/*tra-2* mRNA complexes to the cytoplasm should ultimately result in a reduction of nuclear TRA-1. In fact, boosting TRA-2 activity in the cytoplasm comes at a cost of reducing TRA-1's own feminizing activity in the nucleus (Segal et al., 2001). This curious paradox in the feminizing activity of these proteins suggests that the fine regulation of the amount of TRA-1 in the nucleus and TRA-2 expression in the cytoplasm is crucial to correct female development and further supports the view that the pathway evolves multiple ways to filter the upstream signal by the creation of self-regulatory switches (Segal et al., 2001). The necessity of a complex regulation of the X:A signal is not surprising. Because of the binary nature of the pathway, slight changes in the modulation of feminizing versus masculinizing activity of its members can result in a large number of intermediary sexual phenotypes and sterility. The relationship of TRA-1 and TRA-2 at the protein and RNA levels, illustrates the ever-growing trend in the evolution of new regulatory connections in complex genetic networks.

Moreover, can "sub-modules" within the pathway (e.g. *fem* genes, *sdc* genes, etc) be functionally changed without compromising the interpretation of the X:A signal or do we have to assume, for example, that the *fem* genes will somehow always transduce the male information downstream while *tra-2* orthologues will trigger feminization in *Caenorhabditis*? Though at this moment there is little data to test these possibilities (see Appendix B), it is clear that the simple presence of orthologues does not prove a similar role in a given pathway. A classic example of that is seen in bacteria. Despite sharing a large number of genes in the regulation of chemotaxis, the role of these genes in *Escherichia coli* and *Bacillus subtilis* pathways is surprisingly different. For example, the response to the same attractant can result in inhibition of *E. coli* CheA kinase or its activation in *B. subtilis* (Rao et al., 2004). Conversely, the apparent lack of sequence similarity between orthologues could hide strong conservation of ternary structures of these proteins. Compared to the primary structures, distant homologies between proteins of different species are more apparent in the 3-dimensional structures, where the functional constraint that evolution works upon ultimately lies (Creighton 1993). Until the 3-dimensional structures of sex-determining proteins are known, caution should be taken in assuming functional properties based solely on sequence similarities. Therefore, a scenario where the topology of the sex determination pathway has changed to accommodate the adoption of the correct binary fate in response to a conserved X:A signal should not be dismissed.

Of particular interest is the general evolutionary trend that determines the "acceptable" order of changes to be expected in the evolution of a pathway across time. That becomes important if one considers that the only viable changes are those that do not immediately affect the overall functionality of a given pathway. According to Wilkins (1995), pathways are built from bottom to top. That is to say the function of proteins downstream from a conserved step should be equally conserved, a natural consequence in biological systems where the source of new members is the pathway itself (e.g. gene duplication). This retrograde mode of pathway "growth" predicts that new functional units will likely arise upstream from previously established steps (Horowitz 1945). Hence, the evolutionary constraint for changes in proteins downstream from a conserved step is much greater than upstream from it. However, in the germline sex determination pathways of *C. elegans* and *C. briggsae*, the conserved roles of TRA-2 and TRA-3 contrasts with the lack of functional conservation in the downstream step mediated by the *fem* genes. In order to interpret the evolutionary significance of this change (e.g. whether the XX germline function of the *fem* genes are ancestral or acquired specifically in the *C. elegans* lineage), the role of the *fem* genes in other *Caenorhabditis* species must be analyzed in the light of recent phylogenetic data (Kiontke et al., 2004).

4.10 TRA-2 as a center for the evolution of new regulatory interactions

I provided here evidence for both conservation and functional changes in proteins involved in the nematode sex determination network that occurred since the split from the common ancestor of *C. elegans* and *C. briggsae*. Though the characterization of TRA-1 roles in *C. briggsae* XX and X0 spermatogenesis is still needed, an important aspect observed so far is the relative conservation of TRA-1 and TRA-2 functions in the soma and germline sex determination. This contrasts with the functional divergence of other members of the pathway (e.g. *fem-2, gld-1*). As noted before, this conservation is achieved despite great sequence divergence. The preservation of TRA-1 function can be easily explained by its role in transcriptional regulation. Explaining the preservation of TRA-2 activity in determining the female fate, especially in view of the divergence of the downstream *fem* genes (see above) is less obvious. Given the topology of TRA-2 in the pathway, locked in the interface between the non-cell-autonomous and cell-autonomous domains, a reasonable explanation is that it has been selected as a control center for the transduction of the sex determination signal. Support for that is observed in the various binding partners of TRA-2: TRA-3, FEM-3, HER-1, TRA-1 (TRA-2ic and *tra-2* mRNA) and GLD-1 (*tra-2* mRNA) (see Introduction). The coordination of extracellular inputs, processing and transduction of the signal to its intracellular connections confer a strategic, and possibly highly selected, role for TRA-2 in the pathway. In this view, the membrane receptor TRA-2 would represent a second conserved "niche" in the pathway, upstream from the critical transcriptional activities of the final regulator, TRA-1. A pathway with two conserved "poles" could explain certain aspects of the evolution of sex-determining genes observed here. For instance, the proteins in the pathway that show functional differences between *C. elegans* and *C. briggsae* are directly involved with TRA-2 activity, either as targets or regulators:

a) *C. briggsae* TRA-3 has a stronger feminizing activity than its *C. elegans* homologue (this work).

b) *fem-2* (and possibly *fem-1* and *fem-3*) does not direct male germline fate in *C. briggsae* hermaphrodites, though all three genes are essential for spermatogenesis in *C. elegans* (Stothard et al., 2002 and this work)

c) *C. elegans* GLD-1 is a masculinizing gene whose activity is needed for XX spermatogenesis. In *C. briggsae*, GLD-1 is a feminizing gene necessary for oogenesis (Nayak et al., 2004, see Appendix B).

If we consider what is known about the activity of these proteins in *C. elegans*:

a) The feminizing activity of TRA-3 is accomplished by cleaving and releasing TRA-2ic into the cytoplasm.

b) The masculinizing activity of the complex formed by FEM-1, FEM-2 and FEM-3 is inhibited by the binding of *fem-3* to the intracellular domain of TRA-2 or TRA-2ic.

c) GLD-1 contributes to spermatogenesis in the hermaphrodite by temporarily repressing the translation of *tra-2* mRNA in the L4 larva. Though GLD-1 can bind to the *tra-2* mRNA by itself, the formation of a repression complex requires the presence FOG-2, a protein absent in *C. briggsae* (Nayak et al., 2004).

It looks as if TRA-2 in *C. elegans* and *C. briggsae* is a focal point in the pathway from where new evolving regulatory interactions radiate downstream. More interestingly, this apparent functional flexibility in binding partners has been strongly selected for a feminizing role even amid intense sequence divergence.

Further investigation on the role of *C. briggsae* HER-1 and the existence or not of a TRA-1-TRA-2 regulatory loop in this species, will be important to determine how the TRA-2 connections have evolved.

4.11 Technical considerations and future directions

RNAi has been the strategy of choice in the functional study of homologous genes in *Caenorhabditis* species. By directly targeting a specific transcript, this technique allows to straightforwardly phenocopy the effect of mutant alleles without the aggravation of genetic screens. However, because of the complexity of double-stranded RNA degradation mechanisms and tissue-specific sensibility to RNAi, determining the extent of the knock-down effect becomes a problem. This is particularly critical in the absence of other hypomorphic and null alleles for the locus in question as it was the case of sex-determining genes in non-*C. elegans* nematodes until now. In fact, though RNAi phenotypes for the major sex-determining genes in *C. briggsae* were known before this study, the question of whether they fully represent the null phenotype remained open. Such concerns were stronger when analyzing the effects in the germline, a tissue normally resilient to RNAi in worms. In these instances, the isolation of mutant alleles is necessary. The masculinizing and feminizing alleles isolated in this work have corroborated previously described RNAi phenotypes for sex-determining genes in *C. briggsae*. In particular, RNAi for the *C. briggsae fem* genes and the analysis of suppressors alleles of *tra-2*, which in *C. elegans* always transform a hermaphrodite into a female-only germline, showed the same basic result: suppression of somatic structures but no effect on the onset of XX spermatogenesis. Thus, the mutant phenotype of *tra-2, tra-1* and *fem-2* alleles agree with the knock-down effects of the correspondent RNAi experiments in *C. briggsae*, suggesting that RNAi results for others genes in the pathway are likely significant. Considering the high divergence in these proteins, genetic screens using *C. briggsae* could still be important in identifying new pathway members whose *C. elegans* homologues are either redundant or essential.

Further genetic characterization of the genes involved in sex determination in non-*C. elegans* nematodes is necessary to uncover other specific steps underlying the evolution of this pathway. Importantly, the picture that begins to emerge suggests that germline sex determination is a hub for evolutionary try-outs. The independent rise of hermaphroditism in *C. briggsae* and *C. elegans* exemplifies how the genetic flexibility of the germline differently responded to a strong evolutionary trend towards a self-fertile organism.

A full analysis of *C. briggsae tra-2* suppressors isolated in this study should identify *fem-1* and *fem-3* alleles in this species. Presumably, PCR-deletion screens should provide us with fertile *fem-1* and *fem-3* mutant strains that could be used in genetic crossings to sort out the remaining *tra-2 sup* alleles. Analysis of the germline phenotypes in single and double *fem* mutants should finally resolve whether these genes have any redundant role in the germline of hermaphrodites at all. Furthermore, there is still a chance of other *fem* genes not identified in previous *C. elegans* genetic screens. As with the case of *C. briggsae fem-2*, the *C. elegans* sex determination pathway could have "silent" genes that lost major roles during evolution or became redundant and invisible to screens. If this event was *C. elegans* specific, there is a chance that the mutant *C. briggsae* orthologue would have a particular sex determination phenotype that makes it "visible" to genetic screens. For example, *fem-2* would not have been isolated in a *C. briggsae* forward screen based on its hermaphrodite phenotype. Conversely, as the case of *fog-2* in *C. elegans*, the evolution of new genes with a specific role in the *C. briggsae* germline pathway with no *C. elegans* orthologues cannot be discarded.

Special attention should be given to masculinizing genes acting in the *C. briggsae* germline to allow spermatogenesis in the hermaphrodite. Isolation of *fog-1* and *fog-3* mutations will be essential to understand if these two genes are necessary and sufficient for XX spermatogenesis. Moreover, the translational control of *tra-2* mRNA in the absence of a *fog-2* orthologue and maternal *tra-3* begs the question of how this molecular niche has been filled in *C. briggsae*. Thus, genetic screens targeting mutations that transform *C. briggsae* XX hermaphrodites into females will be necessary to start unraveling how these worms substitute for the activity of the FEM proteins to make sperm. Once the *C. briggsae* sex determination genes acting in the soma and germline are completely dissected, a better picture of the evolution of this molecular pathway in *Caenorhabditis* will arise.

Table 1. *C. elegans* and *C. briggsae* sex determination genes

gene	*C. elegans* *C. briggsae* Protein ID	*C. elegans* protein domain/function	*C. elegans lf* mutant phenotype	*C. briggsae* RNAi x *C. elegans lf* phenotype	*C. briggsae* x *C. elegans lf* phenotypes
Sex determination & dosage compensation					
fox-1	CE25105 CBP00505	RNA-binding	Suppressed lethality of X0 with duplication of X region	-	-
sdc-1	CE03393 CBP12662	Zinc finger TF	Weak XX masculinization	-	-
sdc-2	CE18542 CBP18999	Novel	XX masculinization and lethality	-	-
sdc-3	CE08389 CBP22564	Zinc finger TF	XX masculinization and lethality	-	-
sex-1	CE03323 CBP18903	Nuclear hormone receptor	XX masculinization and dosage compensation defects	-	-
xol-1	CE33915 CBP10365	GHMP kinase	X0 lethal	-	-
Somatic sex determination					
fkh-6	CE03865 CBP14892	Forkhead TF	X0 somatic gonad defects	-	-
mab-3	CE14902 CBP05898	DNA-binding	X0 abnormal tail, feminized intestine	-	-
Somatic and germline sex determination					
fem-1	CE07175 CBP04707	Ankyrin repeats	Complete XX, X0 feminization	No XX GL feminization	-
fem-2	CE02878 CBP03653	Phosphatase (PP2C)	Complete XX, X0 feminization	No XX GL feminization	No XX GL feminization
fem-3	CE02953 CBP11911	Novel	Complete XX, X0 feminization	No XX GL feminization	-
her-1	CE06617 CBP19474	Secretory signal	X0 fertile hermaphrodites	-	-
tra-1	CE28129 CBP17758	Zinc finger TF	XX fertile males	-	XX sterile males
tra-2	CE23546 CBP17144	Membrane receptor	XX sterile intersex	same	same
tra-3	CE16260 CBP24199	Calpain protease	XX sterile intersex	-	same
Germline sex determination					
fog-1	CE27480 CBP18142	RNA-binding (CPEB)	XX,X0 oocytes only	-	-
fog-2	CE23287 -	F-box	XX oocytes only	-	-
fog-3	CE07874 CBP09064	Member of Tob family	XX,X0 oocytes only	same	-
gld-1	CE14096 CBP05692	RNA-binding (STAR)	XX oocytes only	XX sperm only	-
nos-1	CE01614 CBP11611	Nanos-like	XX excess sperm	-	-
nos-2	CE05121 CBP13015	Nanos-like	XX excess sperm	-	-
nos-3	CE19224 CBP00229	Nanos-like	XX excess sperm	-	-

gene	*C. elegans* *C. briggsae* Protein ID	*C. elegans* protein domain/function	*C. elegans lf* mutant phenotype	*C. briggsae* RNAi x *C. elegans lf* phenotype	*C. briggsae* x *C. elegans lf* phenotypes
fbf-1	CE20960 CBP14598	RNA-binding	XX excess sperm	-	-
fbf-2	CE01916 CBP14598	RNA-binding	XX excess sperm	-	-
mog-1	CE01927 CBP16676	RNA helicase (DEAH)	XX sperm only	-	-
mog-2	not cloned	-	XX sperm only	-	-
mog-3	not cloned	-	XX sperm only	-	-
mog-4	CE15592 CBP04901	RNA helicase (DEAH)	XX sperm only	-	-
mog-5	CE01889 CBP00651	RNA helicase (DEAH)	XX sperm only	-	-
mog-6	CE01596 CBP00627	Peptidyl-prolyl cis-trans isomerase	XX sperm only	-	-

Adapted from Nayak et al., 2004 and Stothard and Pilgrim 2003.

C. elegans and *C. briggsae* Protein ID – access numbers from Wormbase (http:www.wormbase.org).

GL- germline, TF- transcription factor.

Table 2. PCR primers used in this work

Name	5'- 3' Sequence
Cbtra-2RTCF	cgaggttttatcactggtc
Cbtra-2RTCR	gatgctctcccaggatgat
Cbtra-2GENINCFO	gatcgggtagtcacctatctaac
Cbtra-2GENINRE	cagcaatgaggaatgcaggtaga
EH21*	tgctcccaatacgctgctgggc
EH22*	cgagatcatcggtcggccaggg

* Obtained from Eric Haag, University of Maryland

Table 3. *C. briggsae* non-sex determination strains used in this work

Name	Genotype	phenotype	purpose	Obtained from
AF16	+	wild type strain	Outcrosses	CGC
BW1850	*mih-3(s12902)*	Self-progeny with 8% males	Make *fem-B;mih-3* strains	CGC
BC1974	*cby-4 (s1272)III*	"chubby" worms	Linkage mapping CIII	Dr. D.Baillie*
BC5983	*cby-15 (sy5148)II*	"chubby" worms	Linkage mapping CII	Dr. D.Baillie
BC5914	*cby-7 (sy5027)IV*	"chubby" worms	Linkage mapping CIV	Dr. D.Baillie
-	*unc (sy5422)III*	paralyzed worms	Linkage mapping CIII	Dr. D.Baillie
PB107	*mih-1(bd102);cby-3(bd101)X*	Cby worms, 14% males in self-progeny	Linkage mapping X	CGC
HC189	integrated array *sid-2::gfp; pEON2 [rol-6(su1006)]*	"rollers", GFP-positive intestinal cells	*tra-3(ed24ts)*; *rol-6* (*tra* complementation)	Dr. C. Hunter**

* Dr. David Baillie, Simon Frasier University

**Dr. Craig Hunter, Harvard University

Table 4. *C. briggsae* sex determination strains used in this work

gene(s)	strain name	genotype	SD phenotypes of homozygotes	
			16°C	25°C
tra-1[*]	CP38	*tra-1(nm2) + / + let (nm28)*	XX: somatically males, intersex GL (sterile) X0: *not observed*	same as 16°C
tra-2[*]	CP20	*tra-2(nm1) + / + cby (nm4)*	XX: intersex X0: wild type	same as 16°C
tra-2	DP297	*tra-2(ed23ts)*	wild type	XX: intersex X0: wild type
tra-3	DP298	*tra-3(ed24ts)*	XX: ~60% wild type, 40% intersex X0: wild type	XX: intersex X0: wild type
fem-2	CP36	*fem-2(nm27)*	XX: wild type X0: transformed hermaphrodites (fertile)	same as 16°C
fem-B	DP366	*fem-B(ed30)*	XX: wild type X0: intersex GL, weak tail feminization (sterile)	same as 16°C
fem-B-mih-3	DP367	*fem-B(ed30);mih-3(s12902)*	same as above	same as above
tra-2;tra-3	DP368	*tra-2(ed23ts) cby-15(sy5148); tra-3(ed24ts)*	XX: ~60% wild type, 40% intersex X0: wild type	XX: intersex X0: wild type
tra-2;fem-2	DP369	*tra-2(ed23ts) cby-15(sy5148); fem-2(nm27)*	XX: wild type X0: transformed hermaphrodites (fertile)	same as 16°C
tra-2;fem-A	DP373[#]	*tra-2(ed23ts);fem-A(ed31)*	XX: 30% with male GL X0: low penatrance of weak somatic feminization (fertile)	same as 16°C
tra-2;fem-B	DP374[#]	*tra-2(ed23ts);fem-B(ed30)*	XX: wild type X0: intersex GL, weak tail feminization (sterile)	XX: intersex tail, wild type GL (self-fertile) X0: intersex GL, weak tail feminization (sterile)
tra-2;fem-B	DP370	*tra-2(ed23ts) cby-15(sy5148); fem-B(ed30)*	same as above	same as above
tra-3;fem-B	DP371	*tra-3(ed24ts);fem-B(ed30)*	same as above	same as above
tra-3;fem-2	DP372	*tra-3(ed24ts);fem-2(nm27)*	XX: wild type X0: transformed hermaphrodites (fertile)	same as 16°C
tra-3;fem-C	DP375[#]	*tra-3(ed24ts);fem-C(ed32)*	wild type	wild type
tra-2;fem-C[**]	*Not maintained*	*tra-2(ed23ts) cby-15(sy5148); fem-C(ed32)*	wild type	XX: *tra-2*-like X0: *not observed*
tra-1;tra-2[**]	*Not viable*	*tra-1(nm2); tra-2(ed23ts) cby-15(sy5148)*	XX: *tra-1*-like X0: *not observed*	XX: *tra-1*-like X0: *not observed*
tra-1;tra-3[**]	*Not viable*	*tra-1(nm2);tra-3(ed24ts)*	XX: *tra-1*-like X0: *not observed*	XX: *tra-1*-like X0: *not observed*

[*] Balanced *tra* strains (Eric Haag), [#] Isolated in the *tra-2* or *tra-3* suppression screens, [**] not available as strains (sterile or not maintained), GL-germline; SD- sex determination

Table 5. Complementation of *tra* alleles

alleles	*nm1*	*nm2*	*ed24*	*ed23*
ed23	**-**	+	**+**	-
ed24	+	**+**	-	
nm2	+*	-		
nm1	-			

(+) complementation (wild type worms); (-) no complementation (*tra* worms). Symbols in bold indicate crosses made. *The allelic relationship between *nm1* and *nm2* was based on mapping data (Eric Haag, personal communication). The remaining relationships were derived from the interpretation of crosses made; *nm1* and *ed23* do not complement each other. *ed24* complements both *ed23* and *nm2*. Therefore three complementation groups are represented (*nm1-ed23*; *ed24*; *nm2*).

Table 6. Fem-2 phenotypes

genotypes	*C. elegans* *fem-2(e2105)**	*C. briggsae* *fem-2(nm27)* CP36	*C. briggsae* *fem-2(nm27);tra-2(ed23ts)* DP369
XX		*phenotypes at 25°C*	
fem-2/+ **m+z+**	hermaphrodite	hermaphrodite	intersex (*tra-2-like*)
fem-2/fem-2 **m-z-**	female	hermaphrodite	hermaphrodite
fem-2/+ **m-z+**	hermaphrodite	hermaphrodite	intersex soma, fertile (47%)
fem-2/fem-2 **m+z-**	hermaphrodite	hermaphrodite	hermaphrodite
X0		*phenotypes at 25°C*	
fem-2/+ **m+z+**	male	intersex GL	intersex GL
fem-2/fem-2 **m-z-**	female (25°C) intersex (20°C)	hermaphrodite**	hermaphrodite
fem-2/+ **m-z+**	male	intersex GL	intersex GL
fem-2/fem-2 **m+z-**	sterile (25°C) male (20°C)	*not observed*	*not observed*

*Hodgkin 1986, **Eric Haag, personal communication, GL: germline

Table 7. Fem-B phenotypes

genotypes	*C. briggsae* *fem-B(ed30)* DP366	*C. briggsae* *fem-B(ed30);tra-2(ed23ts)* DP374
XX	*phenotypes at 25°C*	
fem-B/+ **m+z+**	hermaphrodite	intersex (*tra-2-like*)
fem-B/fem-B **m-z-**	hermaphrodite	intersex tail, GL rescued (fertile)
fem-B/+ **m-z+**	hermaphrodite	intersex (*tra-2-like*)
fem-B/fem-B **m+z-**	hermaphrodite	intersex tail, GL rescued (fertile)
X0	*phenotypes at 25°C*	
fem-B/+ **m+z+**	intersex GL (sterile), weakly feminized soma	intersex GL (sterile), weakly feminized soma
fem-B/fem-B **m-z-**	same as above	same as above
fem-B/+ **m-z+**	same as above	same as above
fem-B/fem-B **m+z-**	*not observed*	*not observed*

GL: germline

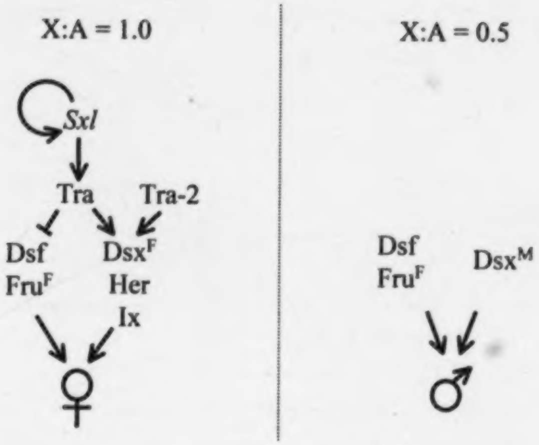


Figure 1. Somatic sex determination in *Drosophila melanogaster*. In females (XX), a X:A of 1.0 results in transcription of *Sxl* (*Sex lethal*) from a female-specific promoter. Sxl controls the splicing of *Sxl* RNA transcribed from a second non-sex-specific promoter, resulting in the production of more Sxl protein in females. In addition, Sxl produces functional *tra* (*transformer*) transcripts while repressing the translation of *msl-2* (*male-specific lethal*) needed for dosage compensation. The Tra proteins alter the splicing of *dsx* (*doublesex*) and *fru* (*fruitless*). Somatic female differentiation depends on the activity of the female Dsx isoform (Dsx^F), Her (Hermaphrodite) and Ix (Intersex) proteins. In XY animals (X:A = 0.5), *Sxl* transcription occurs exclusively from the non-sex-specific promoter. In the absence of preexisting Sxl protein, these transcripts retain a premature stop codon that prevents functional Sxl from being produced. Consequently, no functional Tra protein is present in males, and the male isoforms of Dsx and Fru are expressed. Male somatic differentiation relies on Dsx^M ,Fru^M and Dsf activity. (Zarkower 2001; Schutt and Nothiger 2000).

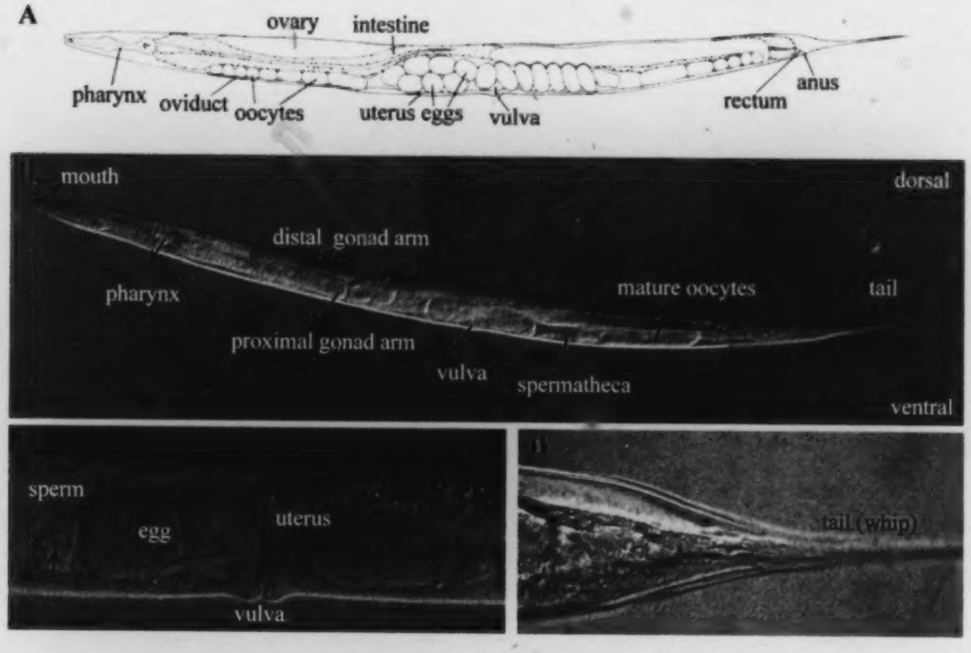

A
ovary
intestine
pharynx
oviduct
oocytes
uterus
eggs
vulva
rectum
anus
mouth
dorsal
distal gonad arm
pharynx
mature oocytes
tail
proximal gonad arm
vulva
spermatheca
ventral
sperm
egg
uterus
vulva
tail (whip)

Figure 2. Hermaphrodite worms. **(A)** Schematic drawing of the *C. elegans* hermaphrodite (adapted from Sulston and Horvitz 1977). **(B)** Photograph of an adult *C. briggsae* hermaphrodite. **(C)** Photograph of the *C. briggsae* hermaphrodite uterus region **(D)** Photograph of the *C. briggsae* hermaphrodite tail. Anatomic positions of animal in all photographs in this thesis are as follow: left-anterior; right-posterior; top-dorsal; bottom-ventral.

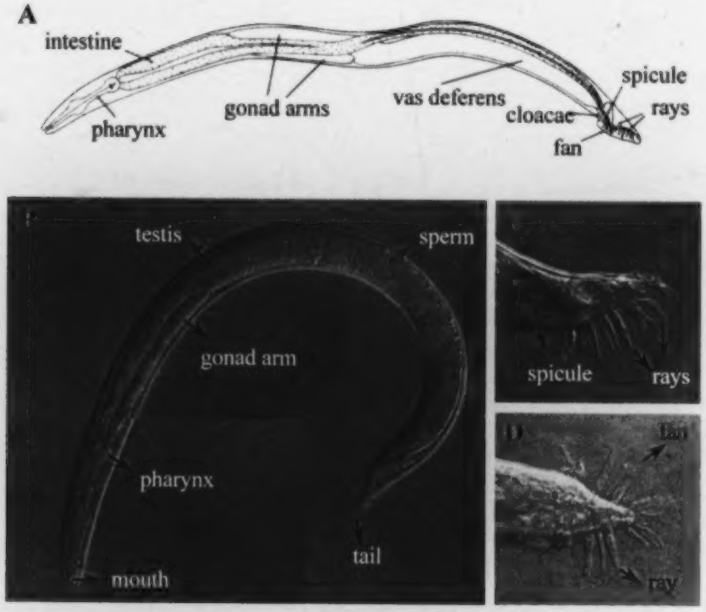

A
intestine
pharynx
gonad arms
vas deferens
cloacae
spicule
rays
fan
testis
sperm
gonad arm
pharynx
mouth
tail
spicule
rays
D
fan
ray

Figure 3. Male worms. (**A**) Schematic drawing of the *C. elegans* male (adapted from Sulston and Horvitz 1977). (**B**) Photograph of an adult *C. briggsae* male. (**C**) Lateral and dorsal (**D**) view of the *C. briggsae* male tail.

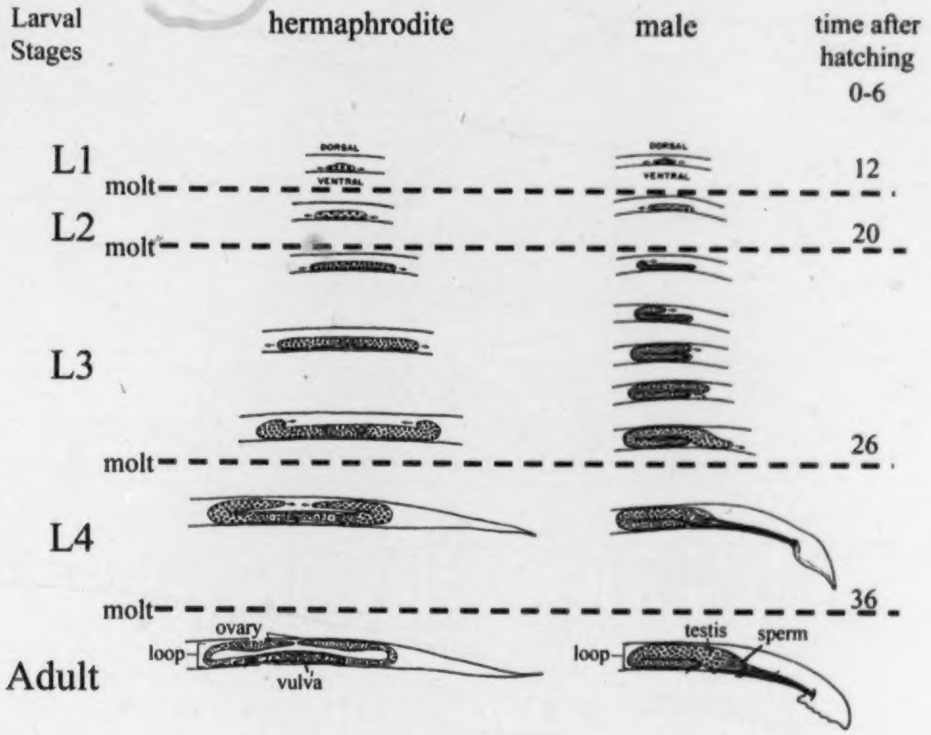

Larval Stages
hermaphrodite
male
time after hatching
0-6
L1
DORSAL
VENTRAL
molt
12
L2
molt
20
L3
molt
26
L4
molt
36
Adult
ovary
loop
vulva
loop
testis
sperm

Figure 4. Development of dimorphism in *C. elegans* (adapted from Klass et al., 1976). Different migration patterns of the somatic gonad cells in males and hermaphrodites starts in L3. The germ cells at this time produce sperm in both XX and X0 worms. During L4, the hermaphrodite germline switches to oogenesis and the male tail starts to form.

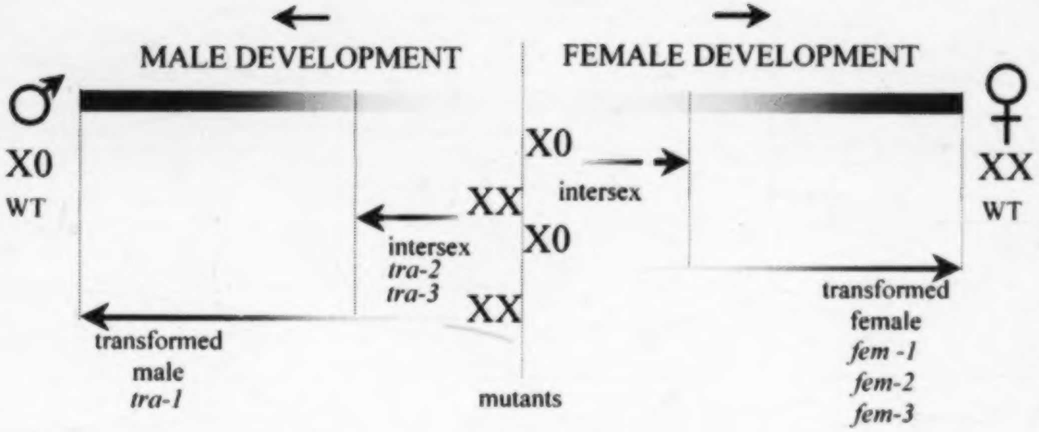
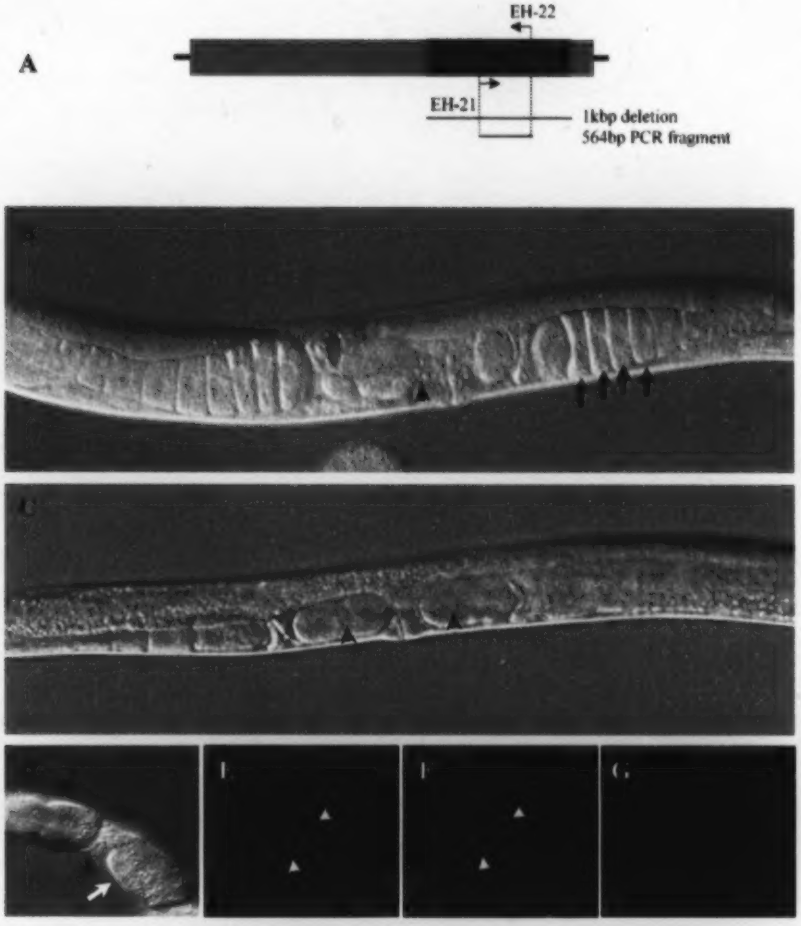

MALE DEVELOPMENT
FEMALE DEVELOPMENT
♂
X0
WT
♀
XX
WT
X0
intersex
XX
intersex
tra-2
tra-3
X0
XX
transformed
male
tra-1
mutants
transformed
female
fem -1
fem-2
fem-3

Figure 5. The binary choice in *C. elegans*. Commitment to wrong developmental program can result in sex-specific phenotypes that contradict the genotype of the worm. The transformation can be complete (e.g. XX *tra-1* worms adopting a male fate and X0 *fem-2* males adopting a female fate) or incomplete (e.g. intersex phenotypes in XX *tra-2* worms). Note that the phenotypic sex can mislead in identifying the genotypic sex. For instance, the phenotype of a XX intersex worm partially adopting a male fate can be very similar to a X0 worm partially adopting a female fate.

Figure 6. *fem-2* mutant strains. (**A**) Schematic representation of *C. briggsae fem-2* genomic region. The 1kbp deletion in *fem-2(nm27)* worms is shown in black. Primers EH-21 and EH-22 that anneal in the deletion region were used to genotype *fem-2(nm27)*. (**B**) DIC photograph of *C. elegans fem-2(b245)* XX females grown at 25°C. In contrast to the *C. briggsae fem-2* mutant animals in **C**, an empty uterus (arrowhead) and accumulation of unfertilized oocytes can be seen in the gonad (arrows). (**C**) DIC photograph of *C. briggsae fem-2(nm27)* hermaphrodite. Note the presence of eggs (arrowhead). (**D**,**E**,**F**,**G**) Immunostaining of the dissected distal gonad of *C. briggsae fem-2(nm27)* hermaphrodite showing the spermatheca region. An oocyte (black arrow) and a fertilized egg (white arrow) are seen to the left and right of the spermatheca, respectively. Sperm cells are present inside spermatheca (arrowheads). These cells have the characteristic condensed sperm nuclei revealed by DAPI staining (**E**) and are recognized by a *C. elegans* monoclonal antibody against the sperm protein SPE56 (**F**). (**G**) Merged photograph of **E** and **F**.


EH-22
A
EH-21
1kbp deletion
564bp PCR fragment
E
F
G

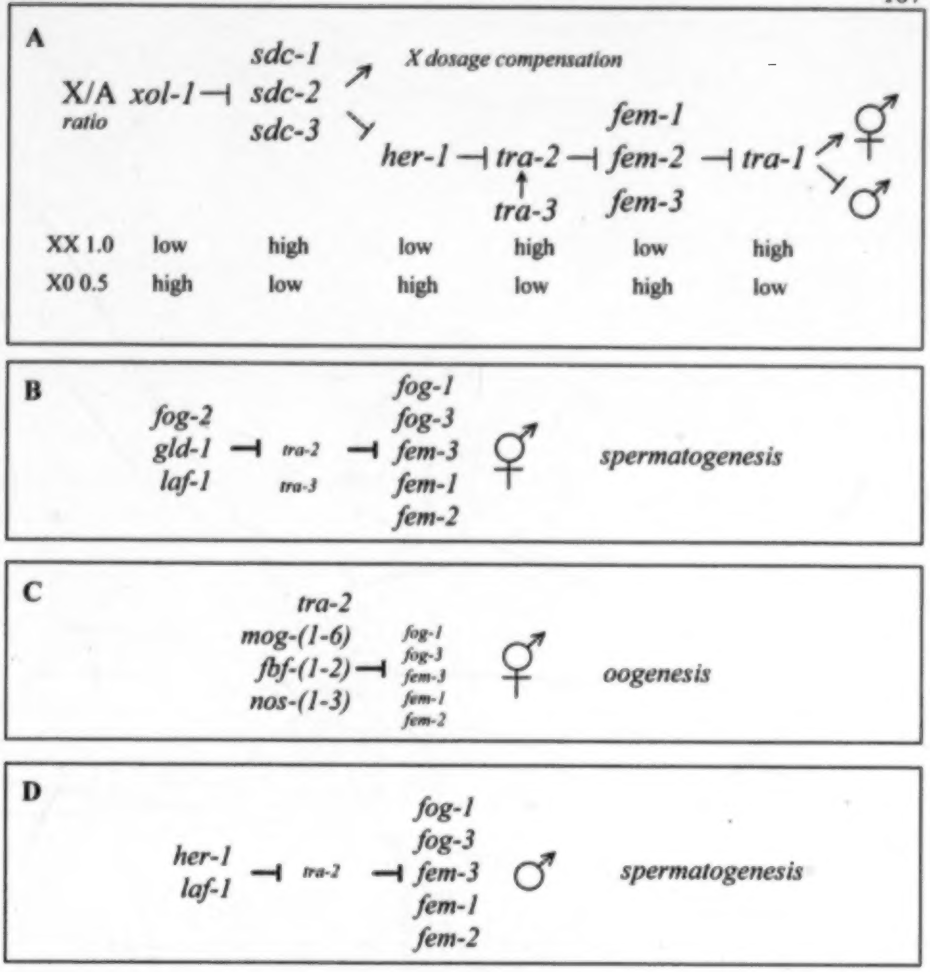

A
X/A ratio
xol-1
sdc-1
sdc-2
sdc-3
X dosage compensation
her-1
tra-2
tra-3
fem-1
fem-2
fem-3
tra-1
⚥
♂
XX 1.0 low high low high low high
X0 0.5 high low high low high low
B
fog-2
gld-1
laf-1
tra-2
tra-3
fog-1
fog-3
fem-3
fem-1
fem-2
⚥
spermatogenesis
C
tra-2
mog-(1-6)
fbf-(1-2)
nos-(1-3)
fog-1
fog-3
fem-3
fem-1
fem-2
⚥
oogenesis
D
her-1
laf-1
tra-2
fog-1
fog-3
fem-3
fem-1
fem-2
♂
spermatogenesis

Figure 7. The sex determination pathway in *C. elegans*. **(A)** Sex determination pathway in the soma. Arrows represent positive interactions, while barred lines indicate negative interactions. The X:A ratio is the initial signal that determines the level of activity (high or low) of male and female promoting factors in each step of the pathway. **(B)** Sex determination pathway in the hermaphrodite germline before the L4 switch (spermatogenesis). Small font represents genes dispensable for the given sexual fate. **(C)** Sex determination pathway in the hermaphrodite after the switch (oogenesis). **(D)** Sex determination pathway in the germline of males.

Figure 8. *tra-2(ed23)* suppressor strains. (**A**) Complementation crosses of 21 *sup* strains. *Sup* hermaphrodites were crossed with *fem-2/+* and *fem-A/fem-A* males and the F1 XX progeny scored. + and - indicate complementation and no-complementation, respectively. Control for allelic mutations for each cross is included in the first two columns. F1 males were scored for feminization of the germline (appearance of "ooids" in the gonad). Y and N indicated feminized and not feminized *sup/+* males, respectively. (**B**) Complementation groups for *tra-2(ed23)* suppressors (top) and *tra-2(ed24)* suppressors (bottom). Asterisks indicate reference strain/allele . The *fem-B* allele *ed30* groups with other *non-fem-2*, *non-fem-A* alleles (*sup* genes). Alleles in this group could potentially represent more than one *sup* gene.

A

tra-2;sup ⚥

	DP369	DP373	DP376	DP377	DP378	DP379	DP380	DP381	DP382	DP383	DP384	DP385	DP386	DP387	DP388	DP389	DP390	DP391	DP392	DP393	DP394	DP395
♂ *fem-2*	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	+	+
fem-A	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FEMINIZED F1 *sup/+* males	Y	N	Y	Y	N	Y	N	Y	N	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	N	Y	Y

B

complementation groups	strain name	**genotype**
fem-A	**DP373***	*tra-2(ed23ts);fem-A(ed31)*
	DP378	*tra-2(ed23ts);fem-A(ed35)*
fem-2	**DP369***	*tra-2(ed23ts) cby-15(sy5148); fem-2(nm27)*
	DP385	*tra-2(ed23ts);fem-2(ed42)*
	DP391	*tra-2(ed23ts);fem-2(ed48)*
	DP392	*tra-2(ed23ts);fem-2(ed49)*
	DP394	*tra-2(ed23ts);fem-2(ed51)*
	DP395	*tra-2(ed23ts);fem-2(ed52)*
fem-B	**DP374***	*tra-2(ed23ts);fem-B(ed30)*
sup	**DP376**	*tra-2(ed23ts);sup(ed33)*
	DP377	*tra-2(ed23ts);sup(ed34)*
	DP379	*tra-2(ed23ts);sup(ed36)*
	DP380	*tra-2(ed23ts);sup(ed37)*
	DP381	*tra-2(ed23ts);sup(ed38)*
	DP382	*tra-2(ed23ts);sup(ed39)*
	DP383	*tra-2(ed23ts);sup(ed40)*
	DP384	*tra-2(ed23ts);sup(ed41)*
	DP386	*tra-2(ed23ts);sup(ed43)*
	DP387	*tra-2(ed23ts);sup(ed44)*
	DP388	*tra-2(ed23ts);sup(ed45)*
	DP389	*tra-2(ed23ts);sup(ed46)*
	DP390	*tra-2(ed23ts);sup(ed47)*
	DP393	*tra-2(ed23ts);sup(ed50)*
fem-C	**DP375***	*tra-[illegible]4ts);fem-C(ed32)*
	DP396	*tra-3(ed24ts);fem-C(ed53)*
	DP397	*tra-3(ed24ts);fem-C(ed54)*

A

Screen #	mutagen	generation	°C	scored F1s	strains	
1	ENU	F2	RT	166	0	
2	EMS	F2	RT	323	0	
3	ENU	F2	RT	274	1	*tra-2(ed23)*
4	ENU	F2	RT	319	1	*tra-3(ed24)*
5	EMS	F2	26°C	258	0	
6	ENU	F2	RT	310	0	
7	ENU	F3	RT	524	0	
8	ENU	F2	RT	414	0	
9	ENU	F3	RT	458	0	
10	ENU	F2	RT	430	0	
				3746 F1 worms	2 *intersex* strains	

B

Screen #	mutagen	generation	°C	scored F1s	strains	
I	EMS	F2	RT	40000	5	*fem-A(ed31)*
II	EMS	F2	RT	40000	7	
III	EMS	F2	RT	40000	5	
IV	EMS	F2	RT	40000	5	
V	EMS	F2	RT	40000	0	
VI	EMS	F2	RT	40000	7	
VII	EMS	F2	RT	80000	15	
VIII	EMS	F2	RT	80000	10	*fem-B(ed30)*
				400000 F1 worms	54 *sup* strains	

C

Screen #	mutagen	generation	°C	scored F1s	strains	
I	EMS	F2	RT	40000	1	
II	EMS	F2	RT	40000	1	
III	EMS	F2	RT	40000	1	*fem-C(ed32)*
				120000 F1 worms	3 *sup* strains	

Figure 9. Mutagenesis screens. Worms were generally selected in F2 progeny. Alleles discussed in this work and the respective screen in which they were isolated are shown in *italics* on the right. (A) Forward screen to isolate *tra* mutants. 10 screens were performed and 2 intersex strains isolated. (B) Screens to isolate *tra-2(ed23)* suppressors. 8 screens yielded 54 sup strains. (C) Suppressor screens of *tra-3(ed24)*. 3 allelic strains were isolated. RT= room temperature (20°C).

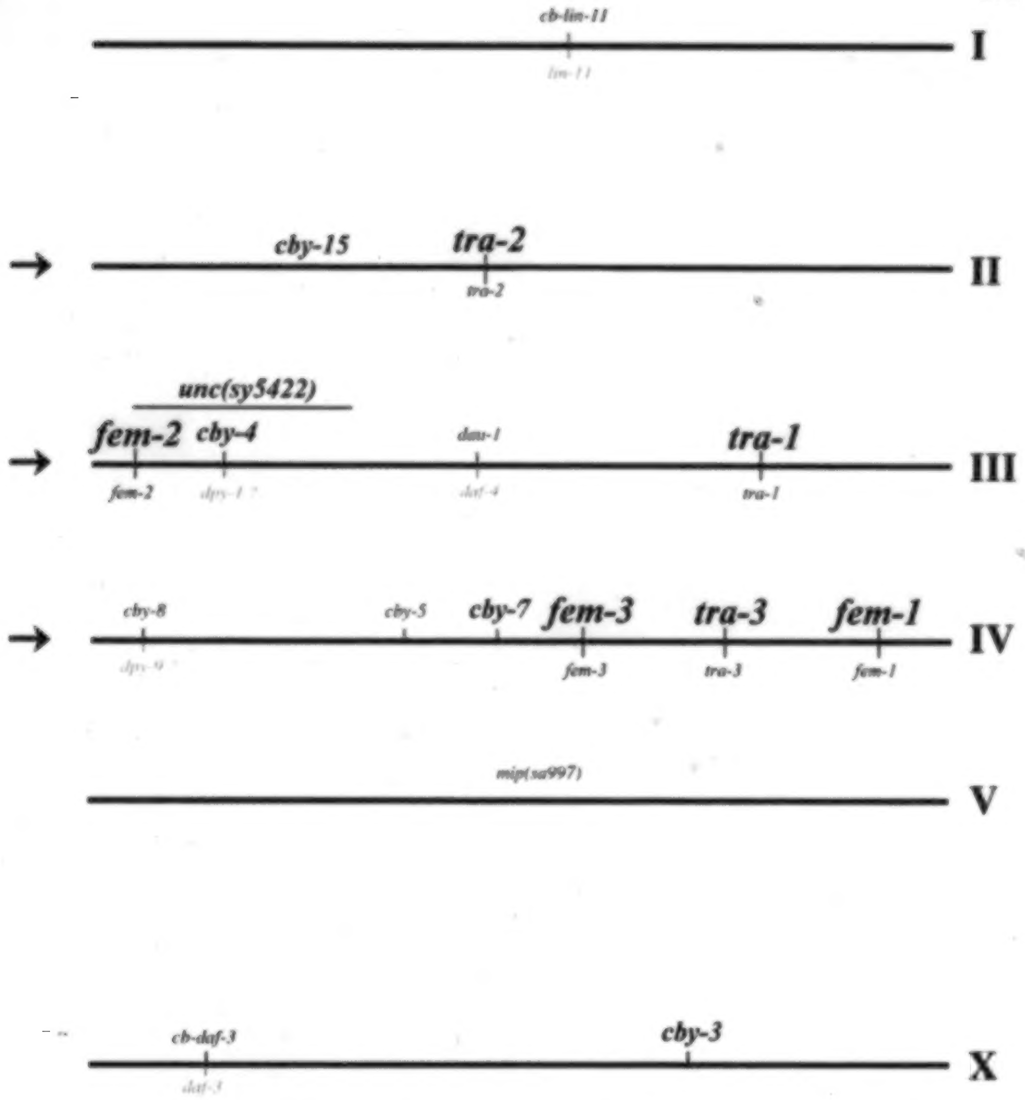

cb-lin-11
lin-11
I
cby-15
tra-2
tra-2
II
unc(sy5422)
fem-2
cby-4
dau-1
tra-1
fem-2
dpy-1 ?
daf-4
tra-1
III
cby-8
cby-5
cby-7
fem-3
tra-3
fem-1
dpy-9 ?
fem-3
tra-3
fem-1
IV
mip(sa997)
V
cb-daf-3
cby-3
daf-3
X

Figure 10. Genetic map of the *C. briggsae* chromosomes. Chromosome numbers and the X chromosome are labeled on the right. The location of *C. briggsae* (*cb*) genes/alleles are shown on the top part of each chromosome and the correspondent *C. elegans* homologue (when characterized) on the bottom. The *C. briggsae* marker genes used in this work are shown in bold. Arrows show chromosomes where the *C.briggsae fem* and *tra* genes (in boxes) are expected to be located, based on synteny with the *C. elegans* orthologues.

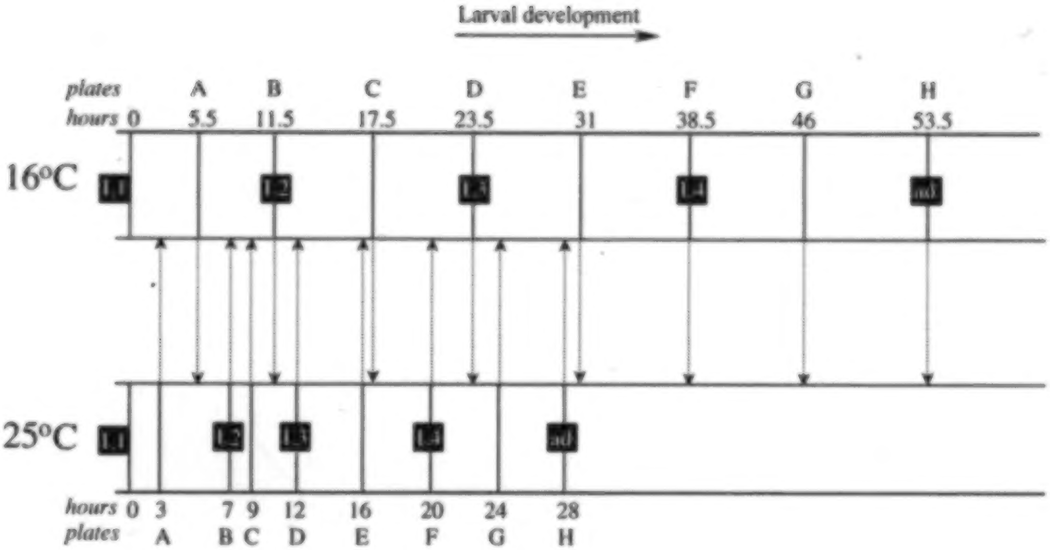

Larval development
plates A B C D E F G H
hours 0 5.5 11.5 17.5 23.5 31 38.5 46 53.5
16°C
L1
L2
L3
L4
ad
25°C
L1
L2
L3
L4
ad
hours 0 3 7 9 12 16 20 24 28
plates A B C D E F G H

Figure 11. Temperature shift experiments. Synchronized worms growing at 16°C or 25°C were shifted once (dotted arrows) either up (16°C to 25°C) or down (25°C to 16°C) at 8 different time points (A to H), spanning all stages of larval development (black boxes, ad=adult). The corresponded time scales are given in hours for both temperatures. Developmental time lines were adopted from *C. elegans* (Byerly et al., 1976). "0" hours corresponded to L1 worms arrested by starvation. After the shift, worms were allowed to grow at the new temperature and adult phenotypes analyzed for tail and germline phenotypes.

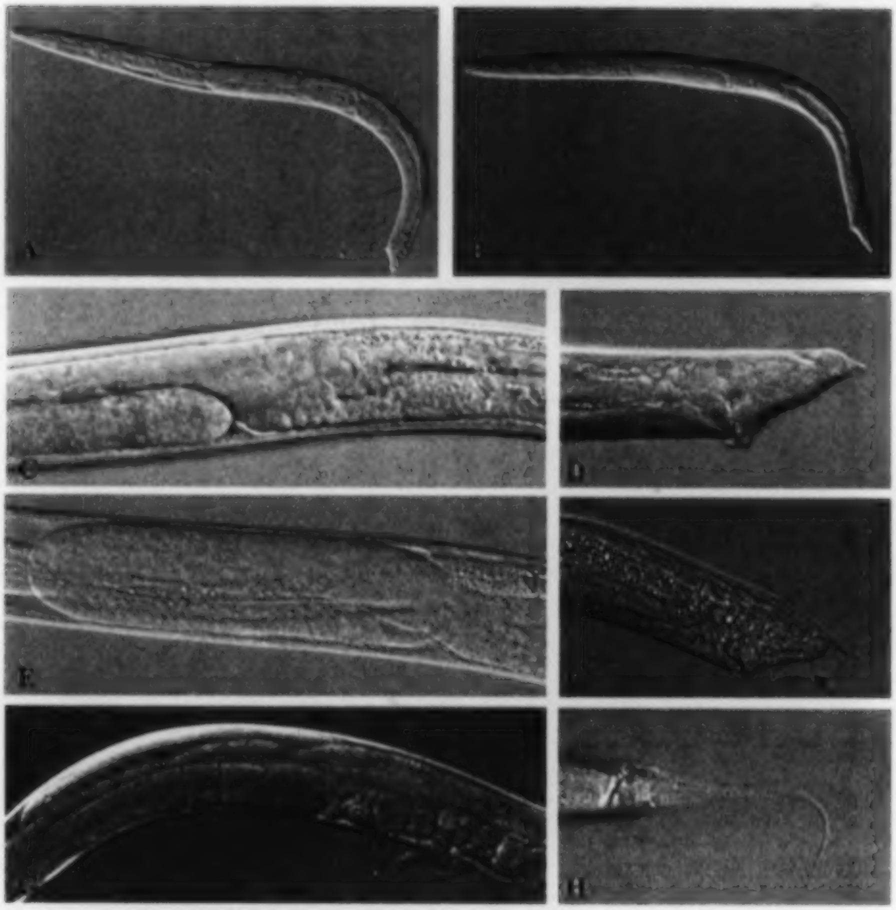


Figure 12. DIC photographs of *C. briggsae tra-2(ed23ts)* (**A**, **E**, **F**, **G**, **H**) and *tra-3(ed24ts)* XX worms (**B**, **C**, **D**). The blunt, incompletely formed male tail, with vestigial rays (arrow) and bursa (**D**, **E**) and the one-arm gonad (**C**, **E**) resulted from somatic masculinization. When grown at 16°C, both strains show reduced Tra phenotype, indicative of temperature sensitive alleles (**G**, **H**).

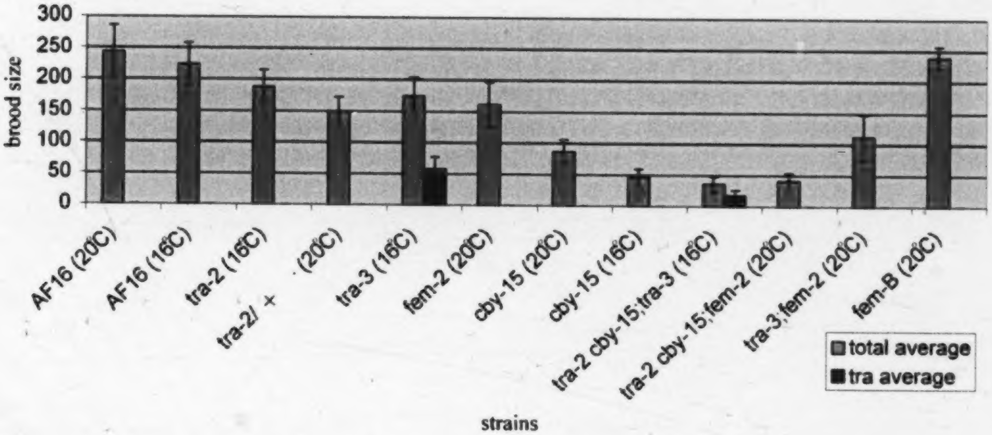


Figure 13. Average brood sizes of selfed hermaphrodites grown either at 20°C or 16°C. The average proportion of tra worms in populations of *tra-3* homozygous hermaphrodites grown at the permissive temperatures is given in red. Alleles: *tra-2 (ed23)*, *tra-3(ed24)*, *fem-2(nm27)*, *cby-15(sy5148)*, *fem-B(ed30)*. Total progenies scored: AF16 20°C (N=10); AF16 16°C (N=11); *tra-2* 16°C (N=7); *tra-2/tra-2+* 20°C (N=9); *tra-3* 16°C (N=15); *fem-2* 20°C (N=15); *cby-15* 20°C (N=17); *cby-15* 16°C (N=9); *tra-2 cby-15; tra-3* 16°C (N=8); *tra-2 cby-15; fem-2* 20°C (N=8); *tra-3;fem-2* 20°C (N=8); *fem-B* 20°C (N=11).

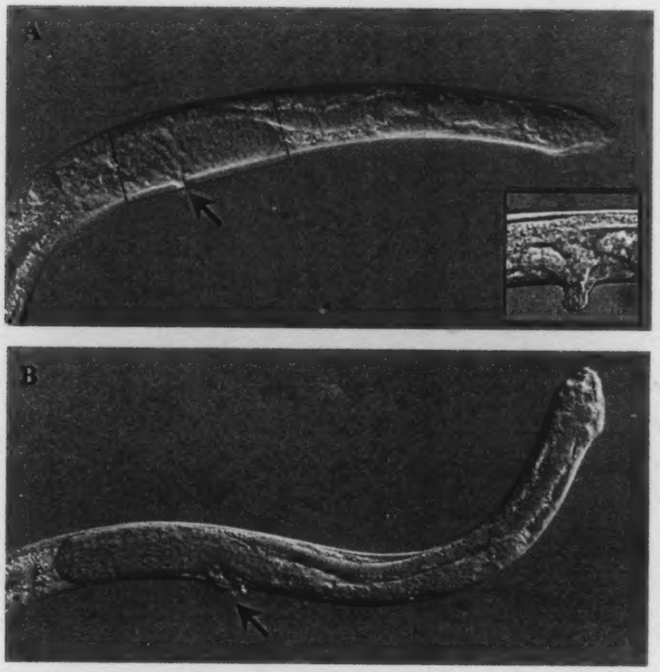


Figure 14. Effects of maternal mRNA contribution of *C. briggsae tra-2* and *tra-3*. **(A)** DIC photograph of a *tra-2/tra-2* worm from *tra-2/+* mothers (m+z-). **(B)** DIC photograph of a *tra-3/tra-3* m+z- XX worm. Somatic and germline masculinization in *tra-2(ed23)* and *tra-2(ed24)* are mostly insensitive to the presence of wild type maternal transcript (m+z-). An exception is the somatic gonad which does not complete the posterior migration, resulting in a ovoid appearance (shaded box in **A**). Since somatic gonad migration influences vulva development (Nelson et al., 1978), m+z- (but only rarely m-z-) worms show an attempt to make a vulva (arrow, box in **A**).

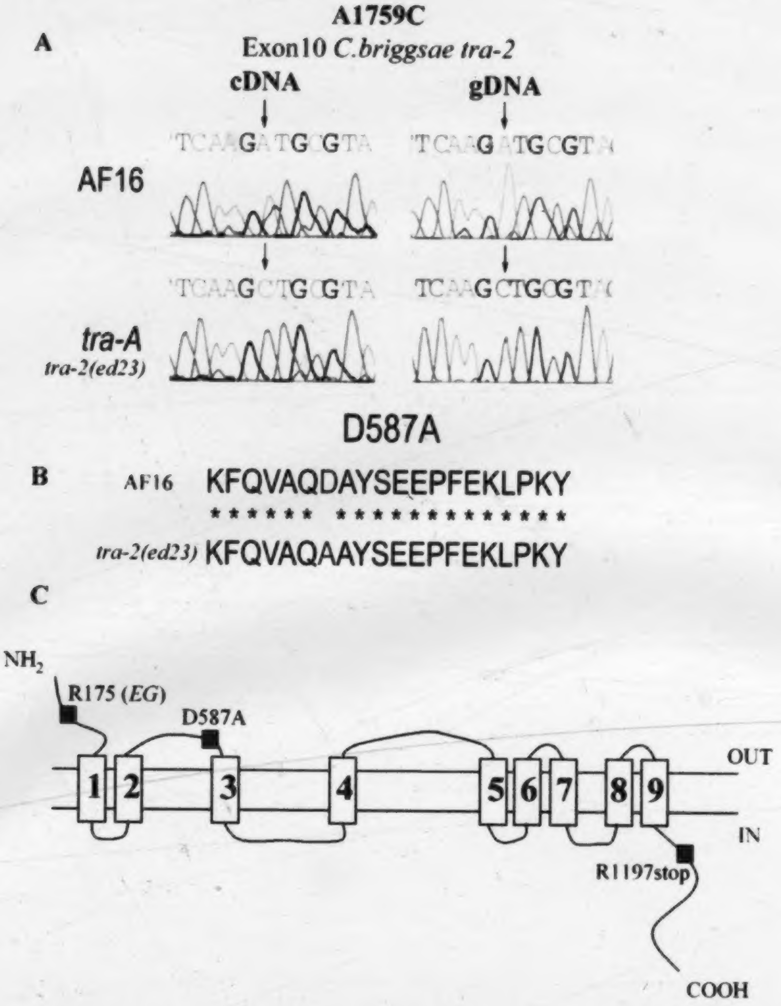

A
A1759C
Exon10 C.briggsae tra-2
cDNA
gDNA
TCAAGATGCGTA
TCAAGATGCGTA
AF16
TCAAGCTGCGTA
TCAAGCTGCGTA
tra-A
tra-2(ed23)
B
D587A
AF16 KFQVAQDAYSEEPFEKLPKY
****** *************
tra-2(ed23) KFQVAQAAYSEEPFEKLPKY
C
NH2
R175 (EG)
D587A
1
2
3
4
5
6
7
8
9
OUT
IN
R1197stop
COOH

Figure 15. *C. briggsae tra-2* alleles. (A) Sequence of cDNA and gDNA regions correspondent to exon 10 of *tra-2* in wild type (AF16) and *tra-2(ed23)* worms. (B) The A1759C mutation in *tra-2(ed23)* worms results in the substitution of a aspartic acid to an alanine in position 587 (D587A). (C) Schematic representation of *C.briggsae* TRA-2. The numbered boxes represent the 9 transmembrane domains. The extracellular amino domain contains the conserved EG site (R175), important for HER-1 binding in *C.elegans* (R177) (Kuwabara 1996a). D587A is located in the first extracellular loop. The mutation in *tra-2(nm1)* worms (R1197stop) is located in the putative FEM-3 binding domain of the intracellular region of the receptor. The sites of both *tra-2* mutations are shown in red.

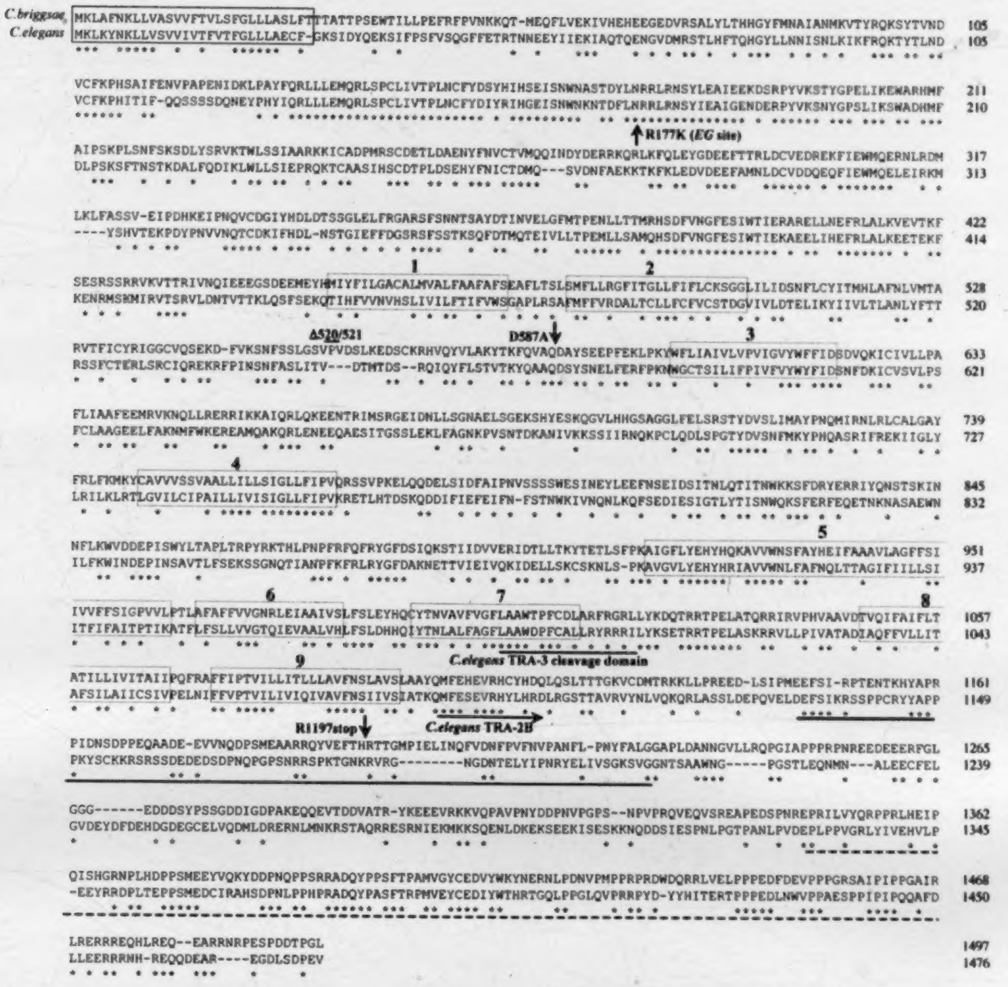

C.briggsae
C.elegans
R177K (EG site)
1
2
Δ520/521
D587A
3
4
5
6
7
8
9
C.elegans TRA-3 cleavage domain
R1197stop
C.elegans TRA-2B

Figure 16. *C. briggsae* and *C. elegans* TRA-2 sequence comparison. Identical residues (43%) are marked by asterisks. The conserved signal peptide sequence (box) in the N-terminal region and transmembrane domains (numbered dotted boxes) are shown. The TRA-3 cleavage domain in TRA-2A and start of *tra-2b* translation is marked in the *C. elegans* sequence. The location of *tra-2* mutant alleles in *C. elegans* (HER-1 binding [EG] site) and *C. briggsae* (D587A and R1197stop) are indicated. Note the particularly low sequence identity in the functionally important intracellular domain, where the *C. elegans* FEM-3 (underlined) and TRA-1 (dashed underline) binding sites are located (Kuwabara 1996b). An *in frame* deletion (Δ520,521) of 6 nucleotides (CCAGTT) in the start of exon 10 was present in both AF16 and *tra-2(ed23)* and differ from the available *C. briggsae* cDNA sequence. Note that the affected residues 520 and 521 are not conserved in *C. elegans*.

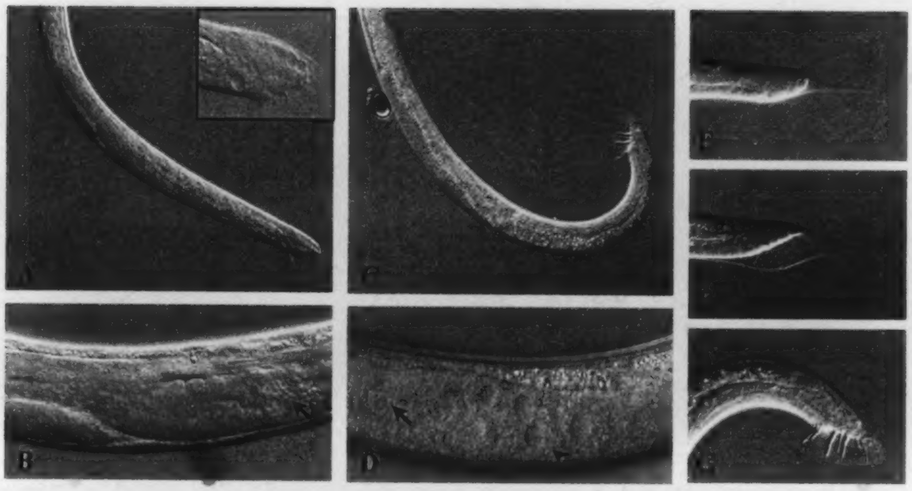
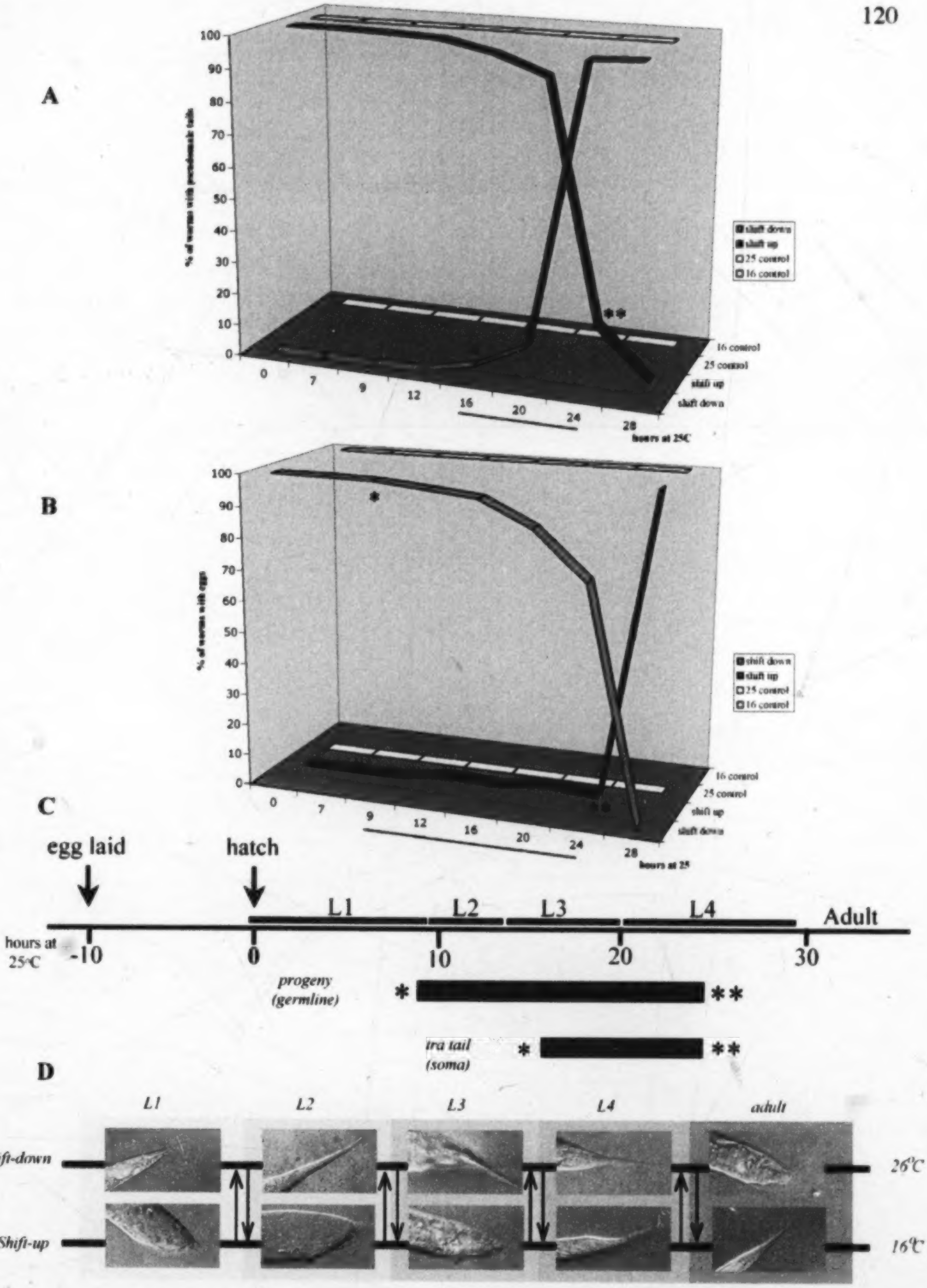


Figure 17. DIC photographs of *tra-2(nm1)* (**A**, **B**) and *tra-1(nm2)* worms (**C**, **D**, **E**, **F**, **G**). Somatic transformation of XX worms into males is complete in *tra-1*(**C**), but not *tra-2* mutants (**A**). As in wild type X0 animals, a male tail starts to develop in XX *tra-1* mutants during early L4 (**F**) and rays elongate during mid L4 (**F**) resulting in a functional male tail after the last molt (**G**). *tra-2* worms have intersex tra tails (box in **A**). The level of germline transformation differs in these mutants. Though the germline of both mutants make sperm (**B**, **D** arrowhead), only *tra-1* mutants produce "ooids" (arrowhead).

Figure 18. Determination of temperature-sensitive periods for male phenotypes in *tra-2.* worms (A) Curves for somatic (*tra* tail) phenotype. (B) Curves for germline (sterility) phenotype. The specific temperature sensitive period (TSP) was assigned as the earliest time in shift-down (*) and the latest time in shift-up (**) experiments in which the phenotype was seen. The length of TSPs for each experiment is shown as a line under the X axis. (C) Soma (red line) and Germline (black line) TSPs for *ed23* during larva development. Asterisks refer to the graphs in A and B. (D) Gradation of tail phenotypes in shifts up and down. Growth at the permissive temperature (16ºC) during the critical developmental time specified by the tail TSP is both necessary and sufficient to rescue the *tra* tail phenotype. Worms shifted to the permissive temperature as late as L3 develop normal hermaphrodite tails. Worms shifted to the restrictive as late as L4, after the critical period, also develop normal tails.


A
% of worms with pseudomale tails
100
90
80
70
60
50
40
30
20
10
0
0
7
9
12
16
20
24
28
hours at 25C
16 control
25 control
shift up
shift down
**
▨ shift down
■ shift up
□ 25 control
▨ 16 control
B
% of worms with eggs
100
90
80
70
60
50
40
30
20
10
0
*
0
7
9
12
16
20
24
28
hours at 25
16 control
25 control
shift up
shift down
▨ shift down
■ shift up
□ 25 control
▨ 16 control
C
egg laid
hatch
L1
L2
L3
L4
Adult
hours at 25°C
-10
0
10
20
30
progeny (germline)
*
**
tra tail (soma)
*
**
D
L1
L2
L3
L4
adult
Shift-down
Shift-up
26°C
16°C

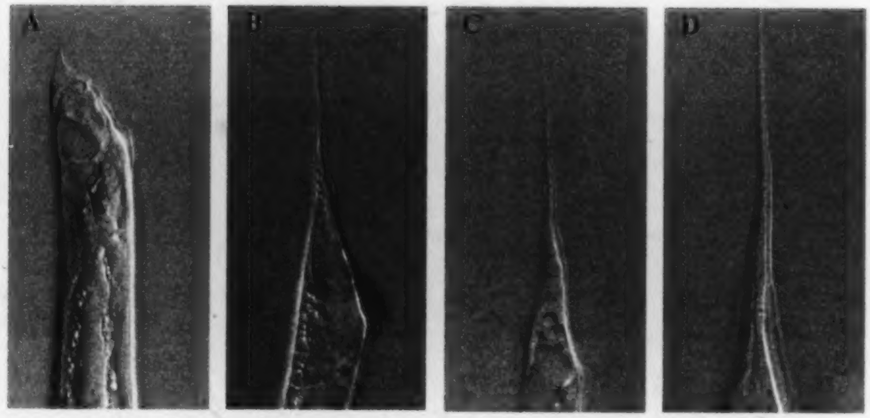


Figure 19. DIC photographs of *tra-2;sup* strains showing the different degrees of suppression of *tra-2* tail phenotype. (**A**) No suppression : *tra-2(ed23)* XX tail. (**B,C**) Intermediate suppression: *sup(ed38)* hermaphrodites. (**D**) Complete suppression : *fem-A(ed31)* hermaphrodite.

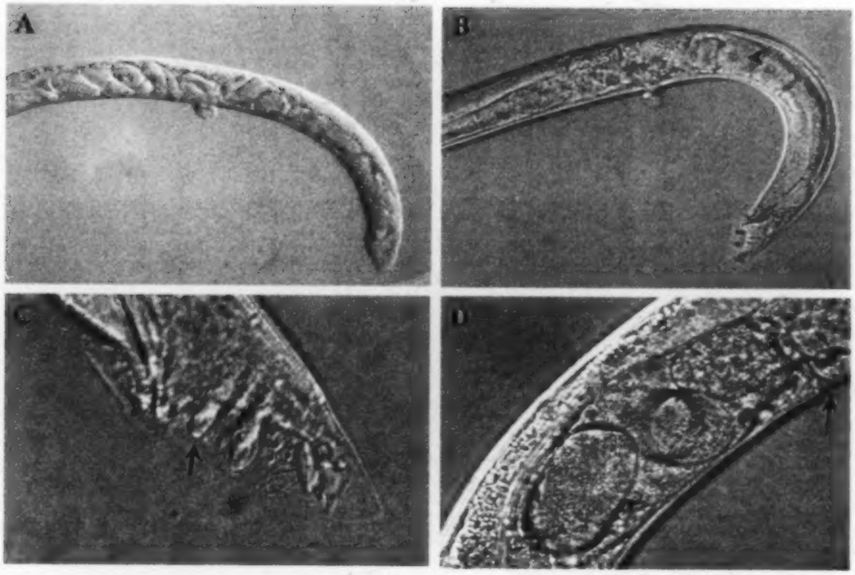


Figure 20. DIC photographs of *fem-B(ed30)* worms. **(A)** *tra-2;fem-B* hermaphrodite (DP374). Note the presence of fertilized eggs in the gonad and the somatic *tra* phenotypes in the tail and vulva. **(B, C, D)** *fem-B* X0 worms (DP366). "Ooids" are seen in the gonad (arrowheads in **B**, **D**), tail rays are abnormal (arrow in **C**) and completely formed vulva can develop (arrow in **C**).

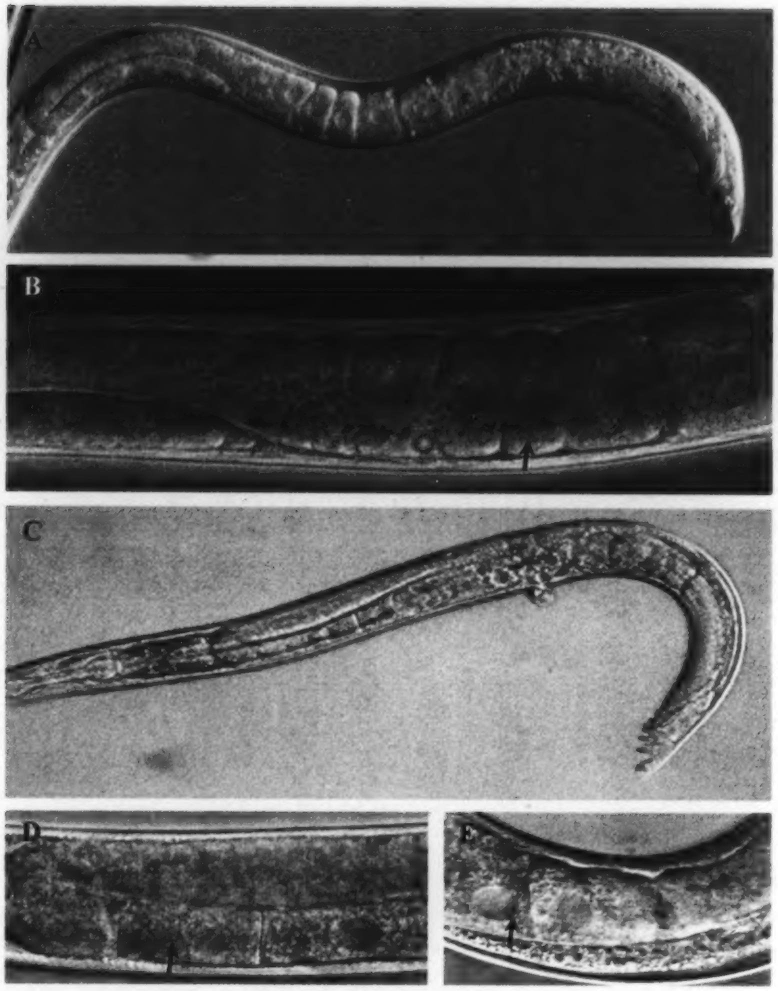
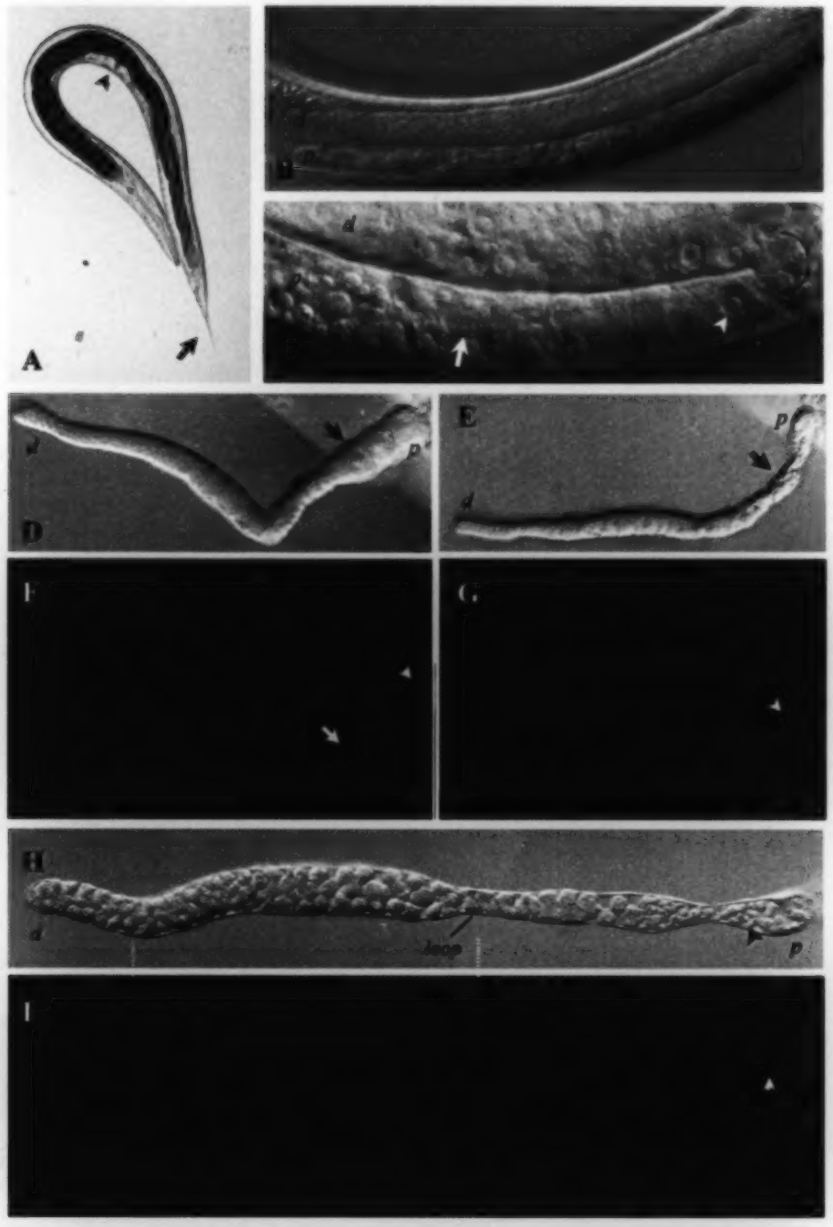


Figure 21. Dose-dependent effect of FEM-2 and FEM-B for X0 germline development. (**A**, **B**) DIC photographs of *fem-2/+* (m+z+) males. (**C**, **D**, **E**) DIC photographs of *fem-B/+* (m+z+) males. The male germline in adult X0 worms shifts to oocyte production. Presence of "ooids" (**B**, **D**, **E** arrows) in otherwise male gonads is seen in heterozygotes and is independent of the female contribution.

Figure 22. DIC (**A**,**B**,**C**,**D**,**E**,**H**) and immunostaining/DAPI (**F**,**G**,**I**) photographs of *tra-2;fem-A* (DP373) hermaphrodite worm and dissected gonads. All somatic defects in the tail (arrow in **A**), vulva (arrowhead in **A**) and somatic gonad (**B**) caused by *tra-2(ed23)* are completely suppressed in these worms. Though the germline of most adult XX worms adopted the correct female fate and underwent oogenesis (arrow in **D**,**F**) after having produced sperm (arrowhead in **F**), some adult worms showed gonads with no mature oocytes (arrow in **E**). Instead, the proximal region of these gonads harbor cells resembling sperm (black arrowheads in **B**,**C**). Initial progression through prophase I is normal as seen by the wild-type pattern of GLD-1 expression (green) in cells in pachytene (upper pictures in **F**,**G** and the domain between white dotted lines in **I**). After reaching the loop (white arrowhead in **C**), differentiating germ cells start to cellularize in an oocyte-like manner as they progress proximally (black arrow in **C**), but next adopt an abnormal round shape (white arrow in **C**) and potentially develop as sperm cells (black arrowhead in **C**). The presence of condensed nuclei in the proximal gonad region (arrowhead in **G**), where mature oocytes should be (arrowhead in **F**), suggests that the germline in these gonads have in fact adopted a male fate. This is further supported by the expression of SPE-56 (red), a male germline marker (upper picture in **I**), in cells undergoing diakinesis (spermatocytes) and differentiated sperm cells (arrowheads in **H**,**I**). *d*- distal gonad; *p*- proximal gonad.


A
B
d
p
D
d
p
E
p
d
F
G
H
d
loop
p
I

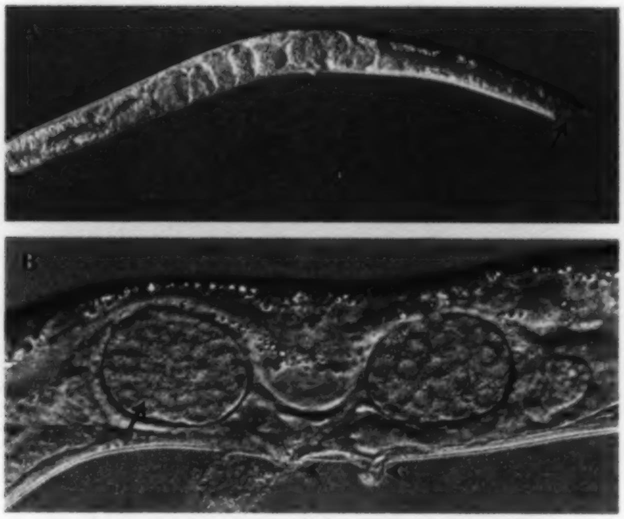


Figure 23. Effects of maternal *C. briggsae fem-2* in suppressing *tra-2* phenotypes. DIC photographs of *tra-2/tra-2;fem-2/+* worms from *tra-2;fem-2* mothers (m-z+). The soma of these worms shows *tra* phenotypes; blunt tail (arrow in **A**) and multivulva (arrowheads in **B**). Partial suppression of the germline masculinization is seen by the presence of fertilized eggs in the gonad (arrow in **B**).

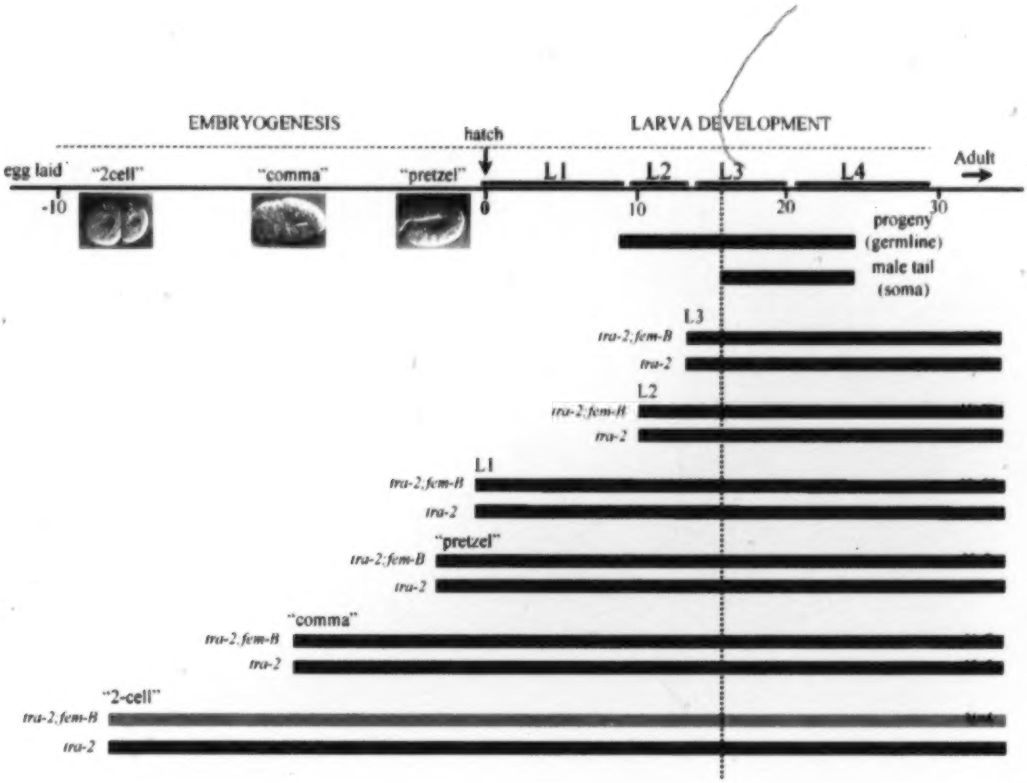


Figure 24. Effects of *fem-B(ed30)* in the TSPs for *ed23*. Shift-up experiments with *tra-2;fem-B* (DP374) and control *tra-2(ed23)* (DP297) embryos and larvae. A time scale (in hours at 25°C) for embryonic and larval development is given at the top. The previously characterized somatic and germline TSP for *ed23* is represented by black lines. The vertical dotted line indicates the boundary for the tail TSP. Somatic and germline phenotypes of XX worms shifted at each time point were scored (total number of tested worms shown in the lines for each shift). Green - wild type soma and germline, red - Tra tail and Tra germline, orange - Tra tail, rescued germline (*tra-2;fem-B* phenotype).

Figure 25. Epistasis analysis and the *C. briggsae* sex determination pathways. **(A)** Genotyping strategy of the *fem-2* locus in double mutants. Homozygous *fem-2* worms lacked a 560bp PCR band spanning the deletion present in *fem-2(nm27)*. **(B)** Interactions tested so far between masculinizing and feminizing genes of the *C. briggsae* sex determination pathway. Order of the genes do not necessarily indicate hierarchy in the pathway. Black lines represent suppression of *tra* phenotypes (soma and/or germline) and dotted lines represent the lack of suppression (soma and germline). Grey lines indicate *tra-1* phenotype for *tra* double mutants. The position of TRA-1 relative to the FEM genes is assumed from *C. elegans* genetics data. **(C, D, E)** Germline sex determination pathways. The roles of genes in grey have not been tested yet. Though spermatogenesis in the X0 germline **(E)** relies on FEM-2 activity, *fem-2* is not required for the onset of hermaphrodite spermatogenesis **(C)**. Exit from spermatogenesis and/or entry in the oogenesis program in the XX germline **(D)** involves the suppression of FEM-2 by TRA-2.

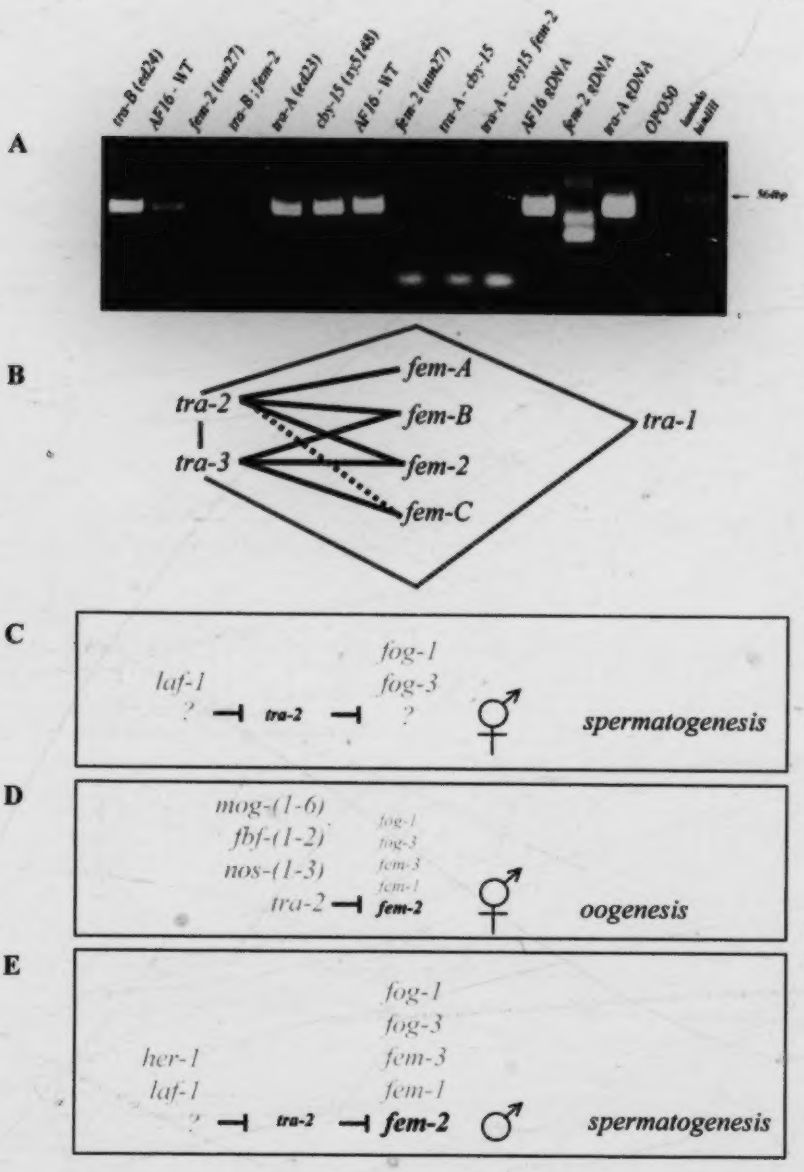
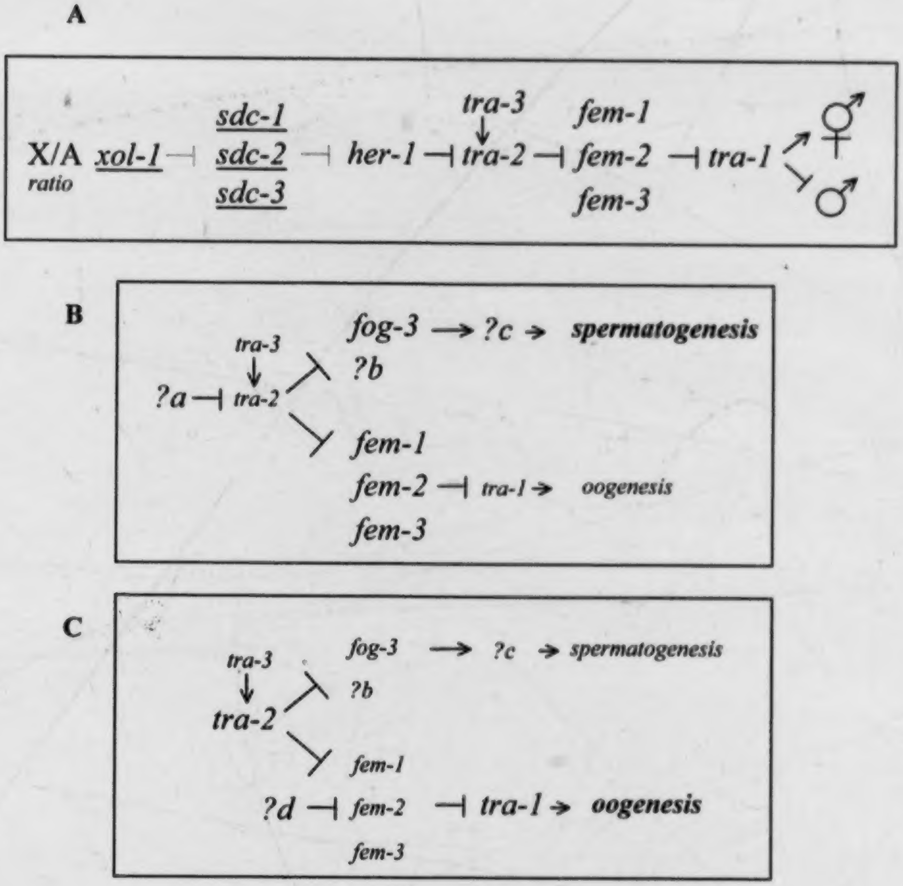

A
tra-B (ed24)
AF16 - WT
fem-2 (nm27)
tra-B ; fem-2
tra-A (ed23)
cby-15 (sy5148)
AF16 - WT
fem-2 (nm27)
tra-A - cby-15
tra-A - cby15 fem-2
AF16 gDNA
fem-2 gDNA
tra-A gDNA
OP50
lambda HindIII
← 564bp
B
tra-2
tra-3
fem-A
fem-B
fem-2
fem-C
tra-1
C
laf-1
?
tra-2
fog-1
fog-3
?
⚥
spermatogenesis
D
mog-(1-6)
fbf-(1-2)
nos-(1-3)
tra-2
fog-1
fog-3
fem-3
fem-1
fem-2
⚥
oogenesis
E
her-1
laf-1
?
tra-2
fog-1
fog-3
fem-3
fem-1
fem-2
♂
spermatogenesis

Figure 26. Possible sex determination pathways in *C. briggsae*. Barred lines represent repression, arrows represent activation. (**A**) Genetics data is consistent with a somatic pathway similar to *C. elegans*. At the moment, no RNAi data or mutant alleles are available for genes in the cell-non-autonomous section of the pathway (underlined genes). (**B**, **C**) Germline sex determination in the hermaphrodite may depend on *tra-2* control of different genes for spermatogenesis and oogenesis (**B**) Spermatogenesis is independent of the *fem* genes but requires an as yet unknown mechanism to transiently repress *tra-2* (?a). *fog-3* and possibly other genes (?b) directly or indirectly (?c) promote spermatogenesis. (**C**) TRA-2 activity could control female fate in two ways; halting spermatogenesis by repressing the *fog* genes and allowing TRA-1 activity by repressing the *fem* genes. In this model, TRA-1 is the final feminizing factor responsible for start of oogenesis. Note that the sperm-oocyte switch regulated by TRA-1 would depend on freeing *tra-2* from "?a" repression, since a downstream mechanism controlling translation of a *fem* gene (?d), as in the case of *C. elegans fem-3*, would not be enough to block the masculinizing signal mediated by the *fog* genes. Moreover, the role of *tra-3* in transducing the female signal involves *tra-2* since *tra-2;sup* alleles also suppress *tra-3*. However, based on the *C. briggsae* mutant phenotype it is likely that TRA-3 activity is more important in this species than in *C. elegans*.


A
X/A ratio
xol-1
sdc-1
sdc-2
sdc-3
her-1
tra-3
tra-2
fem-1
fem-2
fem-3
tra-1
⚥
♂
B
tra-3
?a
tra-2
fog-3
?c
spermatogenesis
?b
fem-1
fem-2
tra-1
oogenesis
fem-3
C
tra-3
tra-2
fog-3
?c
spermatogenesis
?b
fem-1
?d
fem-2
tra-1
oogenesis
fem-3

References

Acampora D, Avantaggiato V, Tuorto F, Barone P, Reichert H, Finkelstein R, Simeone A (1998) Murine Otx1 and *Drosophila* otd genes share conserved genetic functions required in invertebrate and vertebrate brain development. Development 125:1691-1702

Ahringer J, Kimble J (1991) Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3' untranslated region. Nature 349:346-348

Ahringer J, Rosenquist TA, Lawson DN, Kimble J (1992) The *Caenorhabditis elegans* sex-determining gene *fem-3* is regulated post-transcriptionally. EMBO J 11:2303-2310

Akerib CC, Meyer BJ (1994) Identification of X chromosome regions in *Caenorhabditis elegans* that contain sex-determination signal elements. Genetics 138:1-21

Awgulewitsch A, Jacobs D (1992) Deformed autoregulatory element from *Drosophila* functions in a conserved manner in transgenic mice. Nature 358:341-344

Baird SE (2002) Haldane's rule by sexual transformation in *Caenorhabditis*. Genetics 161:1349-1353

Barnes TM, Hodgkin J (1996) The tra-3 sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. EMBO J 15:4477-4484

Barton MK, Kimble J (1990) *fog-1*, a regulatory gene required for specification of spermatogenesis in the germline of *Caenorhabditis elegans*. Genetics 125:29-39

Barton MK, Schedl TB, Kimble J (1987) Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. Genetics 115:107-119

Belfiore M, Pugnale P, Saudan Z, Puoti A (2004) Roles of the *C. elegans* cyclophilin-like protein MOG-6 in MEP-1 binding and germline fates. Development 131:2935-2945

Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77:71-94

Byerly L, Cassada RC, Russel RL (1976) The life cycle of the nematode *Caenorhabditis elegans*. Dev Biol 51:23-33

Callaerts P, Halder G, Gehring WJ (1997) PAX-6 in development and evolution. Annu Rev Neurosci 20:483-532

Carmi I, Kopczynski JB, Meyer BJ (1998) The nuclear hormone receptor SEX-1 is an X-chromosome signal that determines nematode sex. Nature 396:168-173

Chang W, Tilmann C, Thoemke K, Markussen FH, Mathies LD, Kimble J, Zarkower D (2004). A forkhead protein controls sexual identity of the *C. elegans* male somatic gonad. Development 131:1425-1436

Chen PJ, Singal A, Kimble J, Ellis RE (2000) A novel member of the tob family of proteins controls sexual fate in *Caenorhabditis elegans* germ cells. Dev Biol 217:77-90

Chen PJ, Cho S, Jin SW, Ellis RE (2001) Specification of germ cell fates by FOG-3 has been conserved during nematode evolution. Genetics 158:1513-1525

Chin-Sang ID, Spence AM (1996) *Caenorhabditis elegans* sex-determining protein FEM-2 is a protein phosphatase that promotes male development and interacts directly with FEM-3. Genes Dev 10:2314-2325

Clifford R, Lee MH, Nayak S, Ohmachi M, Giorgini F, Schedl T (2000) FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the *C. elegans* hermaphrodite germline. Development 127:5265-5276

Cline T, Meyer B (1996) Vive la difference: Males vs females in flies vs worms. Annu Rev Genet 30:637-702

Coghlan A, Wolfe KH (2002) Four-fold faster rate of genome rearrangement in nematodes than in *Drosophila*. Genome Res 12: 857-867.

Collins CJ, Sedivy JM (2003) Involvement of the INK4a/Arf gene locus in senescence. Aging Cell 2:145-150

Conradt B, Horvitz HR (1998) The C. elegans protein EGL-1 is required for programmed cell death and interacts with Bcl-2-like protein CED-9. Cell 93:519-129

Conradt B, Horvitz HR (1999) The TRA-1A sex determination protein of *C. elegans* regulates sexually dimorphic cell deaths by repressing the *egl-1* cell death activator gene. Cell 98:317-32

Copley RR, Bork P (2000) Homology among βα (8) barrels: implication for the evolution of metabolic pathways. J Mol Biol 303:627-641

Creighton TE (1993) Proteins: Structure and molecular properties. W.H. Freeman and Company, New York.

Creighton TE, Darby NJ (1989) Functional evolutionary divergence of proteolytic enzymes and their inhibitors. Trends Biochem Sci 14:319-324

Cutter AD, Ward S (2005) Sexual and temporal dynamics of molecular evolution in *C. elegans* development. Mol Biol Evol 22:178-188

Davis TL, Meyer BJ (1997) SDC-3 coordinates the assembly of a dosage compensation complex on the nematode X chromosome. Development 124:1019-1031

Dawes HE, Berlin DS, Lapidus DM, Nusbaum C, Davis TL, Meyer BJ (1999) Dosage compensation proteins targeted to X chromosomes by a determinant of hermaphrodite fate. Science 284:1800-1804

de Bono M, Hodgkin J (1996) Evolution of sex determination in *Caenorhabditis*: unusually high divergence of *tra-1* and its functional consequences. Genetics 144:587-595

del Peso L, Gonzalez VM, Inohara N, Ellis RE, Nunez G (2000) Disruption of the CED-9.CED-4 complex by EGL-1 is a critical step for programmed cell death in *Caenorhabditis elegans*. J Biol Chem 275:27205-27211

DeLong L, Plenebisch JD, Klein RD, Meyer BJ (1993) Feedback control of sex determination by dosage compensation revealed through *Caenorhabditis elegans sdc-3* mutations. Genetics 133:875-896

Dierick H, Bejsovec A (1999) Cellular mechanisms of Wingless/Wnt signal transduction. Curr Top Dev Biol 43:153-190

Donahue LM, Quarantillo BA, Wood WB (1987) Molecular analysis of X chromosome dosage compensation in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 84:7600-7604

Doniach T (1986) Activity of the sex-determining gene *tra-2* is modulated to allow spermatogenesis in the *C. elegans* hermaphrodite. Genetics 114: 53-76

Doniach T, Hodgkin J (1984) A sex-determining gene, *fem-1*, required for both male and hermaphrodite development in *Caenorhabditis elegans*. Dev Biol 106:223-235

Ellis RE, Kimble J (1995) The *fog-3* gene and regulation of cell fate in the germline of *Caenorhabditis elegans*. Genetics 139:561-577

Eizinger A, Sommer RJ (1997) The homeotic gene *lin-39* and the evolution of nematode epidermal cell fates. Science 278:452-455.

Fitch DH, Bugaj-Gaweda B, Emmons SW (1995) 18S ribosomal RNA gene phylogeny for some Rhabditidae related to *Caenorhabditis*. Mol Biol Evol 12:346-358

Fitch DH, Thomas WK (1997) Evolution. In: Riddle D, Blumenthal T, Meyer B, Priess J (eds) *C. elegans* II. Cold Spring Harbor Laboratory Press, New York, pp. 815-850

Fodor A, Riddle DL, Nelson FK, Golden JN (1983) Comparison of a new wild type *C. briggsae* with laboratory strains of *C. briggsae* and *C. elegans*. Nematologica 29:203-217.

Francis R, Barton MK, Kimble J, Schedl T (1995a) *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. Genetics 139:579-606

Francis R, Maine E, Schedl T (1995b) Analysis of the multiple roles of *gld-1* in germline development: interactions with the sex determination cascade and the *glp-1* signaling pathway. Genetics 139:607-630

Gallegos M, Ahringer J, Crittenden S, Kimble J (1998) Repression by the 3' UTR of *fem-3*, a sex-determining gene, relies on a ubiquitous *mog*-dependent control in *Caenorhabditis elegans*. EMBO J 17:6337-6347

Goodwin EB, Ellis RE (2002) Turning clustering loops: sex determination in *C. elegans*. Curr Biol 5;12:111-120

Goodwin EB, Hofstra K, Hurney CA, Mango S, Kimble J (1997) A genetic pathway for regulation of *tra-2* translation. Development 124:749-758

Goodwin EB, Okkema PG, Evans TC, Kimble J (1993) Translational regulation of *tra-2* by its 3' untranslated region controls sexual identity in *C. elegans*. Cell 75:329-339

Graham PL, Kimble J (1993) The *mog-1* gene is required for the switch from spermatogenesis to oogenesis in *Caenorhabditis elegans*. Genetics 133:919-931

Graham A, Papalopulu N, Krumlauf R (1989) The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. Cell 57:367-378

Graham PL, Schedl T, Kimble J (1993) More *mog* genes that influence the switch from spermatogenesis to oogenesis in the hermaphrodite germline of *Caenorhabditis elegans*. Dev Genet 14:471-484

Graves LE, Segal S, Goodwin EB (1999) TRA-1 regulates the cellular distribution of the *tra-2* mRNA in *C. elegans*. Nature 399:802-805

Haag ES, Ackerman AD (2005). Intraspecific variation in fem-3 and tra-2, two rapidly coevolving nematode sex-determining genes. Evol Gen (*article in press*).

Haag ES, Doty AV (2005) Sex determination across evolution: connecting the dots. 3:21-24

Haag ES, Kimble J (2000) Regulatory elements required for development of *Caenorhabditis elegans* hermaphrodites are conserved in the *tra-2* homologue of *C. remanei*, a male/female sister species. Genetics 155:105-116

Hansen D, Pilgrim D (1998) Molecular evolution of a sex determination protein: FEM-2 (PP2C) in *Caenorhabditis*. Genetics 149:1353-1362

Hirsh D, Vanderslice R (1976) Temperature-sensitive developmental mutants of *Caenorhabditis elegans*. Dev Biol 49:220-235

Hirth F, Therianos S, Loop T, Gehring WJ, Reichert H, Furukubo-Tokunaga K (1995) Developmental defects in brain segmentation caused by mutations of the homeobox genes orthodenticle and empty spiracles in *Drosophila*. Neuron 15: 769-778

Hodgkin J (1980) More sex-determination mutants of *Caenorhabditis elegans*. Genetics 96:649-664

Hodgkin J (1986) Sex determination in the nematode *C. elegans*: analysis of *tra-3* suppressors and characterization of *fem* genes. Genetics 114:15-52

Hodgkin J (1987) A genetic analysis of the sex-determining gene, *tra-1*, in the nematode *Caenorhabditis elegans*. Genes Dev 1:731-745

Hodgkin J, Barnes TM (1991) More is not better: brood size and population growth in a self-fertilizing nematode. Proc R Soc Lond B Biol Sci 246:19-24

Hodgkin JA, Brenner S (1977) Mutations causing transformation of sexual phenotype in the nematode *Caenorhabditis elegans*. Genetics 86:275-287

Horowitz NH (1945) On the evolution of biochemical syntheses. Proc Natl Acad Scj USA 31:153-157

Hunter CP, Wood WB (1992) Evidence from mosaic analysis of the masculinizing gene *her-1* for cell interactions in *C. elegans* sex determination. Nature 355:551-555

Huynen AM, Gabaldon T, Snel B (2005) Variation and evolution of biomolecular systems: Searching for functional relevance. FEBS let 579:1839-1845

Jager S, Schwartz HT, Horvitz HR, Conradt B (2004) The Caenorhabditis elegans F-box protein SEL-10 promotes female development and may target FEM-1 and FEM-3 for degradation by the proteosome. PNAS 101:12549-12554

Jan E, Motzny CK, Graves LE, Goodwin EB (1999) The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*. EMBO J 18:258-269

Jan E, Yoon JW, Walterhouse D, Lannaccone P, Goodwin EB (1997) Conservation of the *C. elegans tra-2* 3'UTR translational control. EMBO J 16:6301-6313

Jansa SA, Lundrigan BL, Tucker PK (2003) Tests for positive selection on immune and reproductive genes in closely related species of the murine genus mus. J Mol Evol 56:294-307

Jin SW, Kimble J, Ellis RE (2001) Regulation of cell fate in *Caenorhabditis elegans* by a novel cytoplasmic polyadenylation element binding protein. Dev Biol 229:537-553

Jones AR, Francis R, Schedl T (1996) GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. Dev Biol 180:165-183

Jovelin R, Ajie BC, Phillips PC (2003) Molecular evolution and quantitative variation for chemosensory behavior in the nematode genus *Caenorhabditis*. Mol Ecol 12:1325-1337

Kammermeir L, Reichert H (2001) Common developmental genetic mechanisms for patterning invertebrate and vertebrate brains. Brain Res Bull 55:675-682

Kemphues KJ, Strome S (1997) Fertilization and establishment of polarity in the embryo. In: Riddle D, Blumenthal T, Meyer B, Priess J (eds) *C. elegans* II. Cold Spring Harbor Laboratory Press, New York, pp. 335-359

Kennedy S, Wang D, Ruvkun G (2004) A conserved siRNA-degrading Rnase negatively regulates RNA interference in *C. elegans*. Nature 427:645-649

Kimble J, Edgar L, Hirsh D (1984) Specification of male development in *Caenorhabditis elegans*: The *fem* genes. Dev Biol 105:234-239

Kimble J, Sharrock WJ (1983) Tissue-specific synthesis of yolk proteins in *Caenorhabditis elegans*. Dev Biol 96:189-196

Kimble JE, White JG (1981) On the control of germ cell development in *Caenorhabditis elegans*. Dev Biol 81:208-219

King MC, Wilson AC (1975) Evolution at two levels of humans and chimpanzees. Science 11:107-116

Kiontke K, Gavin NP, Raynes Y, Roehrig C, Piano F, Fitch DHA (2004) *Caenorhabditis* phylogeny predicts convergence of hermaphroditism and extensive intron loss. PNAS 15:9003-9008

Kirouac M, Sternberg PW (2003) cis-Regulatory control of three cell fate-specific genes in vulva organogenesis of *Caenorhabditis elegans* and *C. briggsae*. Dev Biol 257:85-103

Klass M, Wolf N, Hirsh D (1976) Development of the male reproductive system and sexual transformation in the nematode *Caenorhabditis elegans*. Dev Biol 52:1-18

Kraemer B, Crittenden S, Gallegos M, Moulder G, Barstead R, Kimble J, Wickens M (1999) NANOS-3 and FBF proteins physically interact to control the sperm-oocyte switch in *Caenorhabditis elegans*. Curr Biol 9:1009-1018

Kuersten S, Segal SP, Verheyden J, LaMartina SM, Goodwin EB (2004) NXF-2, REF-1 and REF-2 affect the choice of nuclear export pathway for *tra-2* mRNA in *C. elegans*. Mol Cell 14:599-610

Kuwabara PE (1996a) A novel regulatory mutation in the *C. elegans* sex determination gene *tra-2* defines a candidate ligand/receptor interaction site. Development 122:2089-2098

Kuwabara PE (1996b) Interspecies comparison reveals evolution of control regions in the nematode sex-determining gene *tra-2*. Genetics 144:597-607

Kuwabara PE, Kimble J (1992) Molecular genetics of sex determination in *C. elegans*. Trends Genet 8:164-168

Kuwabara PE, Kimble J (1995) A predicted membrane protein, TRA-2A, directs hermaphrodite development in *Caenorhabditis elegans*. Development 121:2995-3004

Kuwabara PE, Okkema PG, Kimble J (1992) *tra-2* encodes a membrane protein and may mediate cell communication in the *Caenorhabditis elegans* sex determination pathway. Mol Biol Cell 3:461-473

Kuwabara PE, Okkema PG, Kimble J (1998) Germline regulation of the *Caenorhabditis elegans* sex-determining gene *tra-2*. Develop Biol 204:251-262

Kuwabara PE, Shah S (1994) Cloning by synteny: identifying *C. briggsae* homologues of *C. elegans* genes. Nucleic Acid Res 22:4414-4418

Leuzinger S, Hirth F, Gerlich D, Acampora D, Simeone A, Gehring WJ, Finkelstein R, Furukubo-Tokunaga K, Reichert H (1998) Equivalence of the fly orthodenticle genes and the human OTX genes in embryonic brain development of *Drosophila*. Development 125:1703-1710

Luitjens C, Gallegos M, Kraemer B, Kimble J, Wickens M (2000) CPEB proteins control two key steps in spermatogenesis in *C. elegans*. Genes Dev 14:2596-2609

Lum DH, Kuwabara PE, Zarkower D, Spence AM (2000) Direct protein-protein interaction between the intracellular domain of TRA-2 and the transcription factor TRA-1A modulates feminizing activity in *C. elegans*. Genes Dev 14:3153-3165

Lutfalla G, Crollius HR, Stange-thomann N, Jaillon O, Mogensen K, Monneron D (2003) Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: The class II cytokine receptors and their ligands in mammals and fish. BCM Genomics 4:29-44

Madl EM, Herman RK (1979) Polyploids and sex determination in *Caenorhabditis elegans*. Genetics 93:393-402

Maine EM (2001)RNAi as a tool to understand germline development in Caenorhabditis elegans: use and cautions. Dev Biol 239:177-189

Mathies LD, Schvarzstein M, Morphy KM, Blelloch R, Spence AM, Kimble J (2004) TRA-1/GLI controls development of somatic gonadal precursors in *C. elegans*. Development 131:4333-4343

Matsuo I, Kuratani S, Kimura C, Takeda N, Aizawa S (1995) Mouse Otx2 functions in the formation and patterning of rostral head. Genes Dev 9:2646-2658

Mehra A, Gaudet J, Heck L, Kuwabara PE, Spence AM (1999) Negative regulation of male development in *Caenorhabditis elegans* by a protein-protein interaction between TRA-2A and FEM-3. Genes Dev 13:1453-1463

Meise M, Hilfiker-Kleiner D, Dubendorfer A, Brunner C, Nothiger R, Bopp D (1998) *Sex-lethal*, the master sex-determining gene in *Drosophila*, is not sex-specifically regulated in *Musca domestica*. Development 125:1487-1494

Meyer B, Casson LP (1986) *Caenorhabditis elegans* compensates for the difference in X chromosome dosage between the sexes by regulating transcript levels. Cell 47:871-881

Miller LM, Plenefisch JD, Casson LP, Meyer BJ (1988) *xol-1*: a gene that controls the male modes of both sex determination and X chromosome dosage compensation in *C. elegans*. Cell 55:167-183

Nayak S, Goree J, Schedl T (2004) fog-2 and the evolution of self-fertile hermaphroditism in *Caenorhabditis*. Plos 3:1-15

Nelson GA, Lew KK, Ward S (1978) Intersex – a temperature sensitive mutant of the nematode Caenorhabditis elegans. Dev Biol 66:386-409

Nicoll M, Akerib CC, Meyer B (1997) X-chromosome-counting mechanisms that determine nematode sex. Nature 388:200-204

Nigon V, Dougherty EC (1949) Reproductive patterns and attempts at reciprocal crossing of *Rhabditis elegans Maupas*, 1900, and *Rhabditis briggsae* Dougherty & Nigon, 1949 (*Nematoda:Rhabditidae*). J Exp Zool 112:485-503

Nonet ML, Meyer BJ (1991) Early aspects of *Caenorhabditis elegans* sex determination and dosage compensation are regulated by a zinc-finger protein. Nature 351:65-68

Nusbaum C, Meyer BJ (1989) The *Caenorhabditis elegans* gene *sdc-2* controls sex determination and dosage compensation in XX animals. Genetics 122:579-593

Okkema PG, Kimble J (1991) Molecular analysis of *tra-2*, a sex-determining gene in *C. elegans*. EMBO J. 10:171-176

Pane A, Salvernini M, Delli BP, Polito C, Saccone G (2002) The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. Development 129:3715-3725

Perry MD, Li W, Trent C, Robertson B, Fire A, Hageman JM, Wood WB (1993) Molecular characterization of the *her-1* gene suggests a direct role in cell signaling during *Caenorhabditis elegans* sex determination. Genes Dev 7:216-228

Piekny AJ, Wissmann A, Mains PE (2000) Embryonic morphogenesis in *Caenorhabditis elegans* integrates the activity of LET-502 Rho-binding kinase, MELL-11 Myosin phosphatase, DAF-2 Insulin receptor and FEM-2 PP2C phosphatase. Genetics 156:1671-1689

Pilgrim D, McGregor A, Jackle P, Johnson T, Hansen D (1995) The *C. elegans* sex-determining gene *fem-2* encodes a putative protein phosphatase. Mol Biol Cell 6:1159-117

Pire-daSilva A, Sommer RJ (2004) Conservation of the global sex determination gene tra-1 in distantly related nematodes. Genes Devel 10:1198-1208

Puoti A, Kimble J (1999) The *Caenorhabditis elegans* sex determination gene *mog-1* encodes a member of the DEAH-Box protein family. Mol Cell Biol 19:2189-2197

Puoti A, Kimble J (2000) The hermaphrodite sperm/oocyte switch requires the *Caenorhabditis elegans* homologs of PRP2 and PRP22. Proc Natl Acad Sci USA 97:3276-3281

Rao CV, Kirby JR, Arkin AP (2004) Design and diversity in bacterial chemotaxis: a comparative study in *Escherichia coli* and *Bacillus subtilis*. Plos Biol 2, E49

Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D (2000) *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev 14:2587-2595

Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hirsch B, Hodgkin J, Zarkower D (1998) Evidence for evolutionary conservation of sex-determining genes. Nature 391:691-695

Rhind NR, Miller LM, Kopczynski JB, Meyer BJ (1995) *xol-1* acts as an early switch in the *C. elegans* male/hermaphrodite decision. Cell 80:71-82

Rzhetsky A, Nei M (1992) Statistical properties of the ordinary least –squares, generalized least-squares, and minimum-evolution methods of phylogenetic inference. J Mol Evol 35:367-375

Saccone G, Peluso I, Artiaco D, Giordane E, Bopp D (1998) The *Ceratitis capitata* homologue of the *Drosophila* sex-determining gene *sex-lethal* is structurally conserved, but not sex-specifically regulated. Development 125:1495-1500

Schedin P, Jonas P, Wood WB (1994) Function of the *her-1* gene is required for maintenance of male differentiation in adult tissues of *C. elegans*. Dev Genet 15:231-239

Schedl T, Graham PL, Barton MK, Kimble J (1989) Analysis of the role of *tra-1* in germline sex determination in the nematode *Caenorhabditis elegans*. Genetics 123:755-769

Schedl T, Kimble J (1988) *fog-2*, a germline-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. Genetics 119:43-61

Schutt C, Nothiger R (2000) Structure, function and evolution of sex-determining systems in Dipteran insects. Development 127:667-677

Segal SP, Graves LE, Verheyden J, Goodwin EB (2001) RNA-regulated TRA-1 nuclear export controls sexual fate. Dev Cell 1:539-551

Shen MM, Hodgkin J (1988) *mab-3*, a gene required for sex-specific yolk protein expression and a male-specific lineage in *C. elegans*. Cell 54:1019-1031

Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E (1992) Nested expression domains of four homeobox genes in developing rostral brain. Nature 358:687-690

Skipper M, Milne CA, Hodgkin J (1999) Genetic and molecular analysis of *fox-1*, a numerator element involved in *Caenorhabditis elegans* primary sex determination. Genetics 151:617-631

Schneider RA, Hu D, Helm JA (1999) From head to toe: Conservation of molecular signals regulating limb and craniofacial morphogenesis. Cell Tissue Res 296:103-109

Sokol SB, Kuwabara PE (2000) Proteolysis in *Caenorhabditis elegans* sex determination: cleavage of TRA-2A by TRA-3. Genes Dev 14:901-906

Spence AM, Coulson A, Hodgkin J (1990) The product of *fem-1*, a nematode sex-determining gene, contains a motif found in cell cycle control proteins and receptors for cell-cell interactions. Cell 60:981-990

Stein LD, Bao Z, Blasiar D, Blumenthal T, Brent MR, Chen N, Chinwalla A, Clarke L, Clee C, Coughlan A, Coulson A, D'Eustachio P, Fitch DHA, Fulton LA, Fulton RE, Griffiths-Jones S, Harris TW, Hillier LW, Kamath R, Kuwabara PE, Mardis ER, Marra MA, Miner TL, Minx P, Mullikin JC, Plumb RW, Rogers J, Schein JE, Sohrmann M, Spieth J, Stajich JE, Wei C, Willey D, Wilson RK, Durbin R, Waterston RH (2003) The genome sequence of *Caenorhabditis briggsae*: A platform for comparative genomics. Plos 2:166-192.

Stothard P, Hansen D, Pilgrim D (2002) Evolution of the PP2C family in *Caenorhabditis*: rapid divergence of the sex-determining protein FEM-2. J Mol Evol 54:267-282

Stothard P, Pilgrim D (2003) Sex-determination gene and pathway evolution in nematodes. Bioassays 25:221-231

Streit A, Li W, Robertson B, Schein J, Kamal IH, Marra M, Wood WB (1999) Homologs of the *Caenorhabditis elegans* masculinizing gene *her-1* in *C. briggsae* and the filarial parasite *Brugia malayi*. Genetics 152:1573-1584

Sulston J, Horvitz HR (1977) Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. Dev Biol 56:110-156

Tang KML, Chan SL, Tan KO, Yu UC (2001) The *C. elegans* sex-determining protein FEM-2 and its human homologue, hFEM-2, are Ca2+/Calmodulin-dependent protein kinase phosphatases that promote apoptosis. J Mol Biol 276: 44202-44202.

Tan KM, Chan SL, Tan KO, Yu VC (2001) The *C. elegans* sex-determining protein FEM-2 and its human homologue, hFEM-2, are Ca2+/calmodulin-dependent protein kinase phosphatases that promote apoptosis. J Biol Chem in press

Teichmann SA, Rison SC, Thornton JM, Riley M, Gough J, Chothia C (2001) The evolution and structural anatomy of the small molecule metabolic pathways in *Escherichia coli*. J Mol Biol 311:693-708

Trent C, Purnell B, Gavinski S, Hageman J, Chamberlin C, Wood WB (1991) Sex-specific transcriptional regulation of the *C. elegans* sex-determining gene *her-1*. Mech Dev 34:43-56

Trent C, Tsung N, Horvitz HR (1983) Egg –laying defective mutants of the nematode *Caenorhabditis elegans*. Genetics 104:619-647

True JR, Carroll SB (2002) Gene co-opting in physiological and morphological evolution. Annu Rev Cell Dev Biol 18:53-80

True JR, Haag ES (2001) Developmental system drift and flexibility in evolutionary trajectories. Evol Dev 3:109-119

Villeneuve AM, Meyer BJ (1990) The role of *sdc-1* in the sex determination and dosage compensation decisions in *Caenorhabditis elegans*. Genetics 124:91-114

Walthour CS, Schaeffer SW (1994) Molecular population genetics of sex determination genes: the *transformer* gene of *Drosophila melanogaster*. Genetics 136:1367-1372

Wang S, Kimble J (2001) The TRA-1 transcription factor binds TRA-2 to regulate sexual fates in *Caenorhabditis elegans*. EMBO J 20:1363-1372

Ward S, Roberts TM, Strome S, Pavalko FM, Hogan E (1986) Monoclonal antibodies that recognize a polypeptide antigenic determinant shared by multiple *Caenorhabditis elegans* sperm-specific proteins. J Cell Bio 102:1778-1786

Whitfield LS, Lovell-Badge R, Goodfellow PN (1993) Rapid sequence evolution of the mammalian sex-determining gene *SRY*. Nature 364:713-715

Widmann C, Gibson S, Jarpe MB, Johnson GL (1999) Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Review 79:143-180

Wilkins AS (1995) Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. BioEssays 17:71-77

Wu CI, Li WH (1985) Evidence for higher rates of nucleotide substitutions in rodents than in man. Proc Natl Acad Sci 82:1741-1745

Yi W, Ross JM, Zarkower D (2000) *mab-3* is a direct *tra-1* target gene regulating diverse aspects of *C. elegans* male sexual development and behavior. Development 127:4469-4480

Zarkower D (2001) Establishing sexual dimorphism: conservation amidst diversity? Nat Rev Genet 2:175-185

Zarkower D, Hodgkin J (1992) Molecular analysis of the *C. elegans* sex-determining gene *tra-1*: a gene encoding two zinc finger proteins. Cell 70:237-249

Zarkower D, Hodgkin J (1993) Zinc fingers in sex determination: only one of the two *C. elegans* TRA-1 proteins binds DNA *in vitro*. Nucleic Acids Res 21:3691-3698

Zhang B, Gallegos M, Puoti A, Durkin E, Fields S, Kimble J, Wickens MP (1997) A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germline. Nature 390:477-484

Appendix A

Anti-FEM-2 antibodies

Over 25 years have passed since the first *C. elegans* sex determination mutants were isolated (Hodgkin 2002). During these years, a huge amount of genetic data have accumulated and used to dissect one of the most studied pathways in worms. The history of *C. elegans* sex determination research overlaps with the booming of molecular biology, a fact witnessed by the progressive change in strategies and techniques used over the years to approach questions beyond the scope of genetics. It is undeniable that for all its importance as a differentiation model, the study of sex determination in the nematode stands as a hallmark of developmental genetics research.

For that reason, it is startling how little is actually known about the signal transduction mechanisms that underlies the interpretation of the X:A ratio. In fact, though the main network of genes has been characterized for years now, important gaps still exist in the understanding of the biochemical nature of the signal, in particular in the cell-autonomous section of the pathway.

One of the main reasons for that (besides the disproportional ratio of geneticists to biochemists in the community) is probably related to the lack of good antibodies available. Most antibody studies involve the characterization of over-expressed tagged proteins in worms. Though these experiments are useful insofar as proving certain protein interactions and testing translational control *in vivo*, they cannot be used to make conclusions about the native proteins. Hence, questions like protein localization of wild type and mutant forms, variation of expression during development, etc, are mostly left unanswered.

I attempted to raise antibodies against *C. elegans, C. briggsae* and *C. remanei* FEM-2 peptides to better characterize the function of these proteins in different *Caenorhabditis* species. I reasoned that the availability of antibodies would reinforce the genetic data obtained with the isolation of mutants and could help in characterizing new FEM-2-binding partners previously undetected by genetic screens. A brief description of the strategy used for the production of new GST-FEM-2 fusion proteins, immunization of rabbits, isolation/purification of sera and Western analysis is given below.

Selecting new antigens

Five different FEM-2 regions (2 from *C. elegans*, 1 from *C. briggsae* and 1 from *C. remanei* FEM-2 proteins) were selected as targets for raising rabbit polyclonal antibodies. The peptides encoded by these regions were located in different domains of the proteins and showed different biochemical characteristics and sequence similarity between orthologues (*figure A1, A2* and *A4*):

Region 1 – PPDA (*C. elegans* **p**hosphatase **d**omain) - A 42 residue peptide, highly hydrophobic, located in the conserved phosphatase domain in the C-terminal region of FEM-2.

Region 2 – CECT (***C. elegans*** **C-t**erminal domain) – A 26 residue peptide, highly acidic and fairly conserved among *fem-2* orthologues but distinct from other *C. elegans* PP2C phosphatases.

Region 3 – HYCE, HYCB, HYCR (**Hy**drophilic ***C. e**legans*, ***C. b**riggsae*, ***C. r**emanei* domains) – 41, 40 and 40 residue peptides, respectively. These peptides were located in a hydrophilic pocket in the C-terminal region and showed low sequence homology with each other.

Cloning *C. elegans* PPDA, CECT and HYCE fragments

A list of primers used is given in *table A1*, and a list of constructs in *table A2*.

A modified pGEX (Amersham) vector containing a PCR-introduced *sal-I* site in its MCS was previously constructed to clone the full length 1407bp *C. elegans fem-2* cDNA. The resulting pPD#DH14 vector carries the *C. elegans fem-2* cDNA sequence downstream from GST and can be used to express a GST-FEM-2 fusion protein (Hansen 1999) in bacteria. Petra Jackle Baldwin (1996), in our lab, had previously used this fusion to raise a polyclonal anti-*C. elegans* FEM-2 antibody in rabbits. The final crude serum was further strip-purified against FEM-2 to clean it from GST-cross reacting IgGs. Though the purified serum did recognize GST-FEM-2 fusions (*figure A3*) and a transgenic *fem-2* construct over-expressed in *C. elegans* hermaphrodites (Stothard 2002), results were less clear in Western blots using *C. elegans* lysates and immunostaining experiments.

pPD#DH14 and primers PPDA3'-PPDA5', CECT3'-CECT5', HYCE3'-HYCE5' were used to PCR-amplify *C. elegans fem-2* cDNA fragments encoding PPDA, CECT and HYCE peptides, respectively. For cloning purposes, amplified CECT and HYCE fragments were flanked by *EcoRI* and *BamHI* sites introduced by the primers. PPDA fragments carried *EcoRI* sequences in both ends. PCR fragments were first cloned into pGEM-T (Amersham) and then subcloned into pGEX-1λ, either directionally (HYCE and CECT) or not (PPDA). Clones were subsequently sequenced using an internal pGEX primer (PGEX3P) to check for correct orientation (PPDA) and PCR-introduced mutations.

Cloning *C. briggsae* HYCB and *C. remanei* HYCR fragments

Primers HYCB3'-HYCB5' and HYCR3'-HYCR5' were used to amplify HYCB and HYCR fragments from *C. briggsae* and *C. remanei* full length *fem-2* cDNA cloned into pGEM-T (pPD#PS3-2p801 and pPD#PS1-2p801) (Stothard 2002). Both sets of primers introduced *EcoRI* sites in the amplified fragments. Inserts were cloned into pGEM-T, subcloned into the *EcoRI* site of pGEX-1λ and checked for correct orientation and mutations.

Expressing and purifying GST-fusion proteins in *E.coli*

BL-21 competent *E. coli* were transformed with pPD#CEC6, pPD#CEC7, pPD#CEC8, pPD#CEC9, pPD#CEC10 (*table A2*) and GST-fusion proteins were induced and isolated (*figure A3*). In brief, cells were pre-cultured overnight at 37°C in 10ml 2YT media (0.16g tryptone, 0.1g yeast extract, 0.05g NaCl in 10ml milliQ H_2O) in the presence of ampicilin (Amp) and cloramphenicol (Cam). The following day 5ml of the overnight culture were added to 1l of fresh 2YT (Amp+ Cam+) medium and left to grow for 2 hours at 37°C (OD~600). The culture was induced with 1mM isopropyl-

1-thio-beta-D-galactopyranoside (IPTG) for 4 hours at 37°C. Bacteria were harvested by centrifugation and resuspended into 9.5ml of sterile PBS (4g NaCl, 0.1g KCl, 0.72g Na_2HPO_4, 0.12g KH_2PO_4 in 50ml ddH_2O) on ice. Cells were disrupted by the addition of Triton X-100 (1% final concentration) followed by sonication. 1mM of phenylmethylsulfonyl fluoride (PMSF) (SIGMA) was added to prevent protease activity. Lysates were centrifuged and supernatant collected.

Isolation of GST-fusions was performed at 4°C using glutathione-sepharose 4B columns (Amersham) previously washed with ice-cold PBS. The flow-through was reloaded into the column three times before being discarded. Columns were washed with 15 bed volumes of PBS and fusions eluted from beads in 2mL of 5mM glutathione, 50mM Tris-HCl (pH 8.0) (Amerham). Finally, elutions were washed and concentrated in PBS using a centrifugal concentrator column (Millipore). I also purified GST from empty pGEX plasmids as well as the full length GST-FEM-2 from pPD#DH14 to use as controls for protein sizes in Western blots.

Antisera production

5, 1, 0.1 and 0.01 µl of purified fusion proteins in PBS were separated by electrophoresis together with different concentrations of bovine serum albumen. Gels were stained with 0.05% Coomassie blue and the approximate concentration of fusion protein solution calculated.

About 500mg of purified fusion protein were mixed with 500ml of Freund's incomplete adjuvant (SIGMA) and inoculated intramuscularly in rabbits. Injections were handled by the Biosciences Animal Services at the University of Alberta. Rabbits were previously selected by testing pre-immunosera in Western blots against *C. elegans* lysates. Only rabbits whose sera did not recognize *C. elegans* antigens were further used. Two rabbits for each recombinant protein were inoculated monthly to a total of 7 boost injections before the animals were killed and the final sera collected and stored at –80°C. Five test-bleeds and the final sera from each of the 10 animals were collected between injections and tested in Western blots against the fusion protein used, full length GST-FEM-2 and lysate of the respective *Caenorhabditis* species.

Western Analysis

A variety of different lysis protocols were used. Worms were ressuspended in PBS-1% Triton-X or TBS-T (0.02M Tris-base 0.8% NaCl, 0.3% Tween-20, pH 7.6), or RIPA (10mM Tris-HCl pH 7.5, 0.15M NaCl, 1% NP40, 0.1% Sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1mM PMSF) or "worm lysate buffer" (50mM ethanolamine pH 8.0, 5mM DTT, 2mM EDTA, 1mM PMSF) (Goetinck and Waterston 1994) and either sonicated or initially disrupted using freeze-crack protocols.

SDS-PAGE was performed using approximately 25μg of lysate protein and 1μg of fusion proteins. Gels were blotted onto nitrocellulose membranes (Bio-Rad) at 4°C, 100V for 80 minutes in transfer buffer (5mM Tris, 38mM glycine and 20% methanol). Membranes were stained with Ponceau S (SIGMA) to check for quality of the transfer before being extensively washed with TBS-T. After blocking overnight with 5% skim milk in TBS-T at 4 °C, membranes were incubated with different concentrations of diluted sera (1:500; 1:1000; 1:10.000) in TBS-T for 2 hours at room temperature (or overnight at 4°C). I used an anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) diluted 10.000 times as the secondary antibody. Immunodetection was performed according to the instructions in the ECL kit (Amersham).

To clean some of the sera from other cross-reacting antibodies, I tried several purification methods. Antibodies were precipitated with ammonium sulfate (Harlow and Lane 1988), absorbed in BL-21 or OP50 acetone powder, isolated in protein-A columns (Amersham), affinity-purified in antigen columns or using strips (Jackle-Baldwin 1996). Because the bulk of the recombinant proteins used as antigens were produced as GST-fusions, I also tried to deplete polyclonal sera of anti-GST antibodies by immobilizing anti-GST IgGs in GST columns and strips.

Despite these efforts, I was unable to characterize a FEM-2 specific band in worm lysates (*figure A6*). Future attempts to raise anti-FEM-2 antibodies should focus on antigens that carry a minimum of non-FEM-2 sequence, for example peptides synthesized in vitro or HIS-tag expression systems. Conversely, the use of eukaryotic expression systems (e.g. insect cells, mammalian cell cultures) may improve protein folding and increase the immunogenic capacity of the antigen.

α-*C. elegans* cN-17 antibody

A commercial polyclonal antibody against the amino region (cN-17 fragment) of *C. elegans fem-2* was obtained from Santa Cruz. Despite the low sequence identity in the amino terminal of *C. elegans* and *C. briggsae* FEM-2 (*figure A1*), α-*C. elegans* cN-17 recognizes ~48KDa band in *C. briggsae* wild type lysates but not in lysates of *fem-2(nm27)* worms (CP36) (*figure A5*). In addition, the commercial antibody also recognized the full length GST-FEM-2 fusion protein. As a control for the quality of lysates,I used a polyclonal anti-*C. elegans* GLD-1 antibody provided by Dr. Schedl's lab at the Washington University School of Medicine (*figure A5*).

Table A1. PCR primers (GST-fusion constructs)

Name	5'- 3' Sequence	Enzyme site
HYCE3'	ccttcca**gaattc**tttcacac	*EcoRI*
HYCE5'	ctcagtac**ggatcc**gggcac	*BamHI*
CECT3'	gataaaatcgaccga**gaattc**gg	*EcoRI*
CECT5'	gttgtgatt**ggatcc**ttgcg	*BamHI*
PPDA3'	gacacgtttc**gaattc**attag	*EcoRI*
PPDA5'	ccatccgat**gaattc**gaagc	*EcoRI*
HYCB3'	cccttcca**gaattc**cttcg	*EcoRI*
HYCB5'	gctggg**gaattc**tgggagac	*EcoRI*
HYCR3'	gattctc**gaattc**tttctgac	*EcoRI*
HYCR5'	ctgggaa**gaattc**tccgatg	*EcoRI*
PGEX3P	gagctgcatgtgtcagagg	-

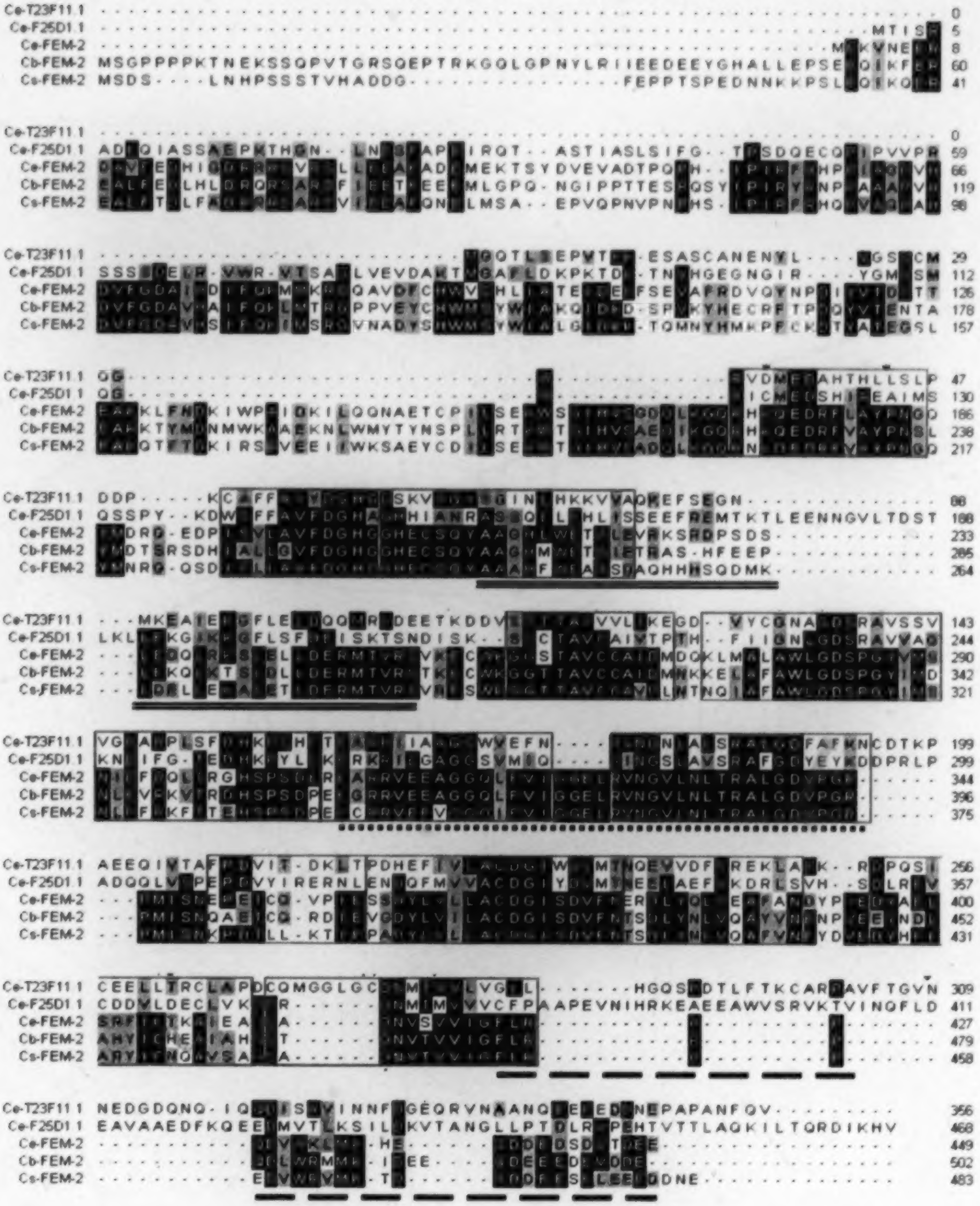
Enzyme sites in primer sequences are marked in bold.

Table A2. Plasmid constructs

Name	Vector	Insert
pPD#DH14[*]	pGEX-1λ	Ce *fem-2* cDNA
pPD#PS3-2p801[**]	pGEM-T	Cb *fem-2* cDNA
pPD#PS1-2p801[**]	pGEM-T	Cr *fem-2* cDNA
pPD#CEC01	pGEM-T	CECT
pPD#CEC02	pGEM-T	PPDA
pPD#CEC03	pGEM-T	HYCE
pPD#CEC04	pGEM-T	HYCB
pPD#CEC05	pGEM-T	HYCR
pPD#CEC06	pGEX-1λ	CECT
pPD#CEC07	pGEX-1λ	PPDA
pPD#CEC08	pGEX-1λ	HYCE
pPD#CEC09	pGEX-1λ	HYCB
pPD#CEC10	pGEX-1λ	HYCR

Ce- *C. elegans* ; Cb – *C. briggsae* ; Cr – *C. remanei*
[*]Hansen 1999 ; [**]Stothard 2002

Figure A1 - Sequence comparison of FEM-2 proteins and two other *C. elegans* PP2C proteins (Ce-T23F11.1 and Ce-F25D1.1). Identical residues are marked in black, protein similarity in grey. Peptides used to generate antiserum are underlined. HYCE, HYCB and HYCR region – double line. *C. elegans* PPDA region - dotted line and CECT in dashed line. Adapted from Stothard et al., 2001.


Ce-T23F11.1
Ce-F25D1.1
Ce-FEM-2
Cb-FEM-2
Cs-FEM-2

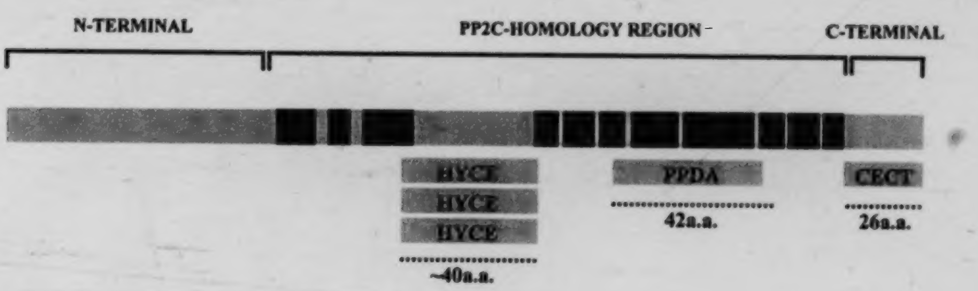

N-TERMINAL
PP2C-HOMOLOGY REGION
C-TERMINAL
HYCE
HYCE
HYCE
~40a.a.
PPDA
42a.a.
CECT
26a.a.

Figure A2- Schematic representation of *C. elegans* FEM-2 and the location of the peptides used for antibody production. Black boxes in the PP2C homology region represent conserved domains.

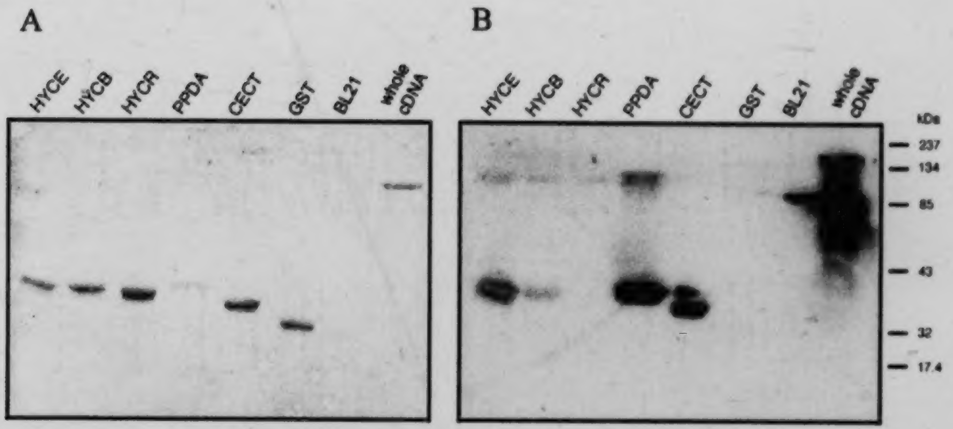

A
B
HYCE
HYCB
HYCR
PPDA
CECT
GST
BL21
whole cDNA
kDa
237
134
85
43
32
17.4

Figure A3 - **A**- Ponceau stained gel of extracted GST-fusion proteins. **B**- Western blot of GST-fusions (gel in A) using a purified anti-*C. elegans* FEM-2 polyclonal antibody raised against the whole protein (whole cDNA lane) (Jackle-Baldwin 1996). BL21 lysates from *E.coli* that did not carry pGEX was used as control for glutathione column-purification (BL21 lane).

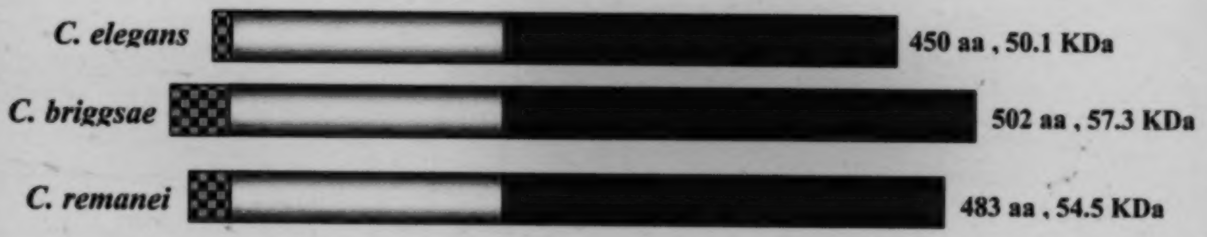

C. elegans
450 aa , 50.1 KDa
C. briggsae
502 aa , 57.3 KDa
C. remanei
483 aa , 54.5 KDa

Figure A4- Schematic comparison of *C. elegans, C. briggsae and C. remanei* FEM-2 proteins. Checked region represents the variable length of the amino-terminal. White region represents the low sequence identity amino domain and black region represents the conserved PP2C-like domain.

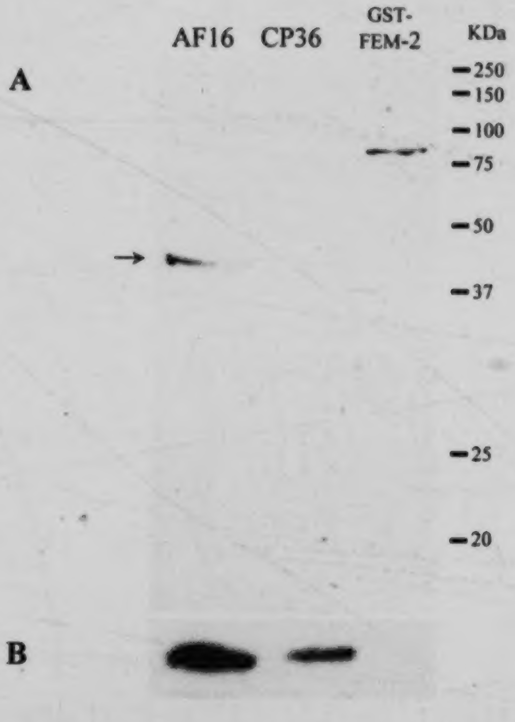
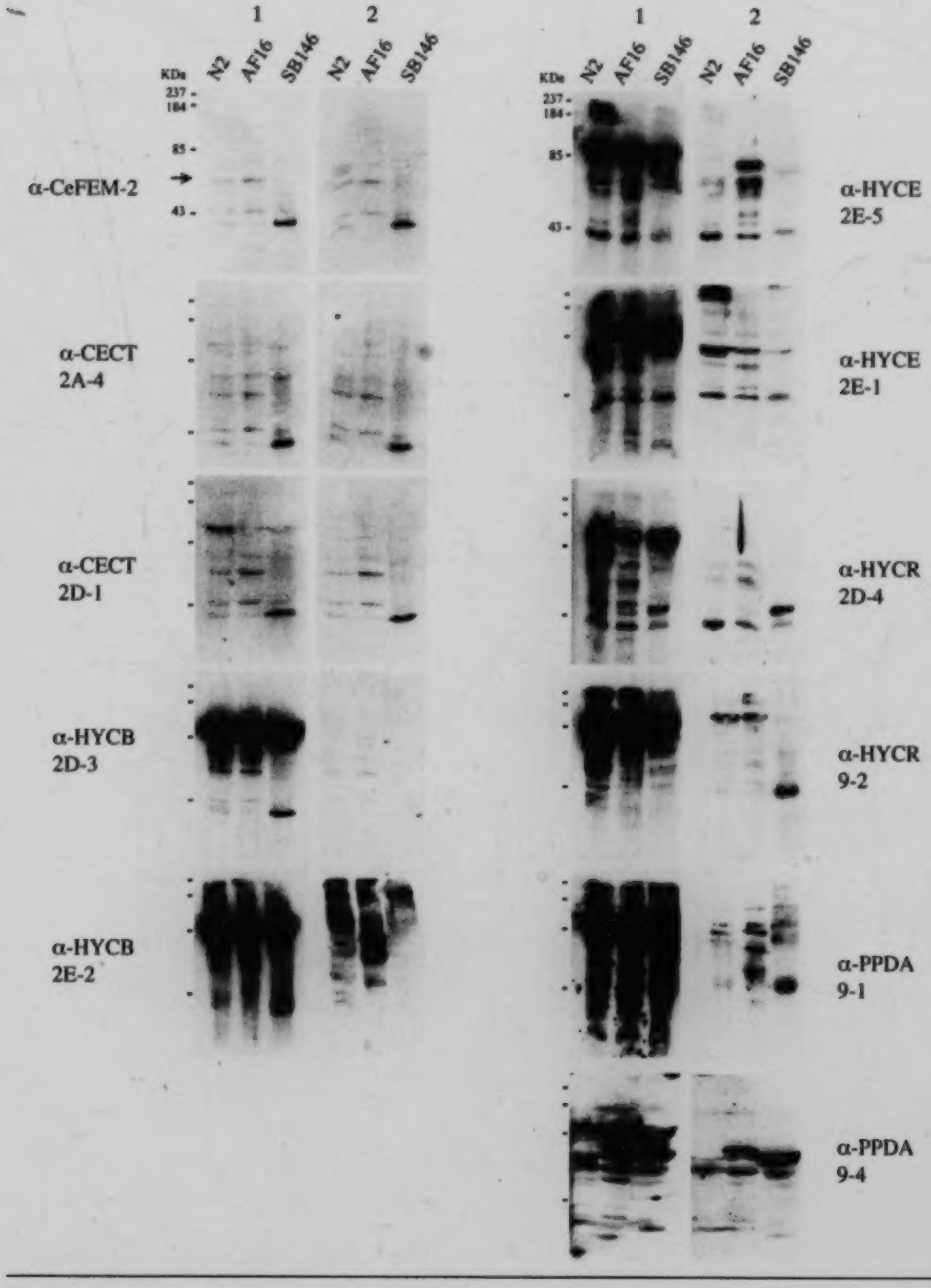

A
AF16
CP36
GST-
FEM-2
KDa
250
150
100
75
50
37
25
20
B

Figure A5- (**A**) Western blot of *C. briggsae* AF16 and CP36 lysates reacted with α-*C. elegans* FEM-2 cN-17 antibody (1.000x diluted) (Santa Cruz). Arrow points to a putative C. briggsae FEM-2 band. A GST fusion containing whole length C. elegans FEM-2 is recognized by α-*C. elegans* FEM-2 cN-17. (**B**) Western blot of the same lysates described above reacted with α*C. elegans* GLD-1 (1000x diluted). A *C. briggsae* 48KDa band is visible in AF16 but not CP36 lysates.

Figure A6- Western blots with *C. elegans* (N2), *C. briggsae* (AF16) and *C. remanei* (SB146) lysates reacted with final 2,000x diluted anti-FEM-2 sera. The correspondent antigen and serum name is given besides each pair of films (two different sera per antigen). Column 1 indicates Western blots performed with crude serum and column 2 indicated Western blots performed with serum pre-incubated with BL-21 acetone powder. The previously raised and purified anti-FEM-2 antibody (Jackle-Baldwin 1996) was used as control (top left pair). The arrow points to the putative *C. elegans* FEM-2 band.


1
2
N2
AF16
SB146
N2
AF16
SB146
KDa
237
184
85
43
α-CeFEM-2
α-CECT 2A-4
α-CECT 2D-1
α-HYCB 2D-3
α-HYCB 2E-2
α-HYCE 2E-5
α-HYCE 2E-1
α-HYCR 2D-4
α-HYCR 9-2
α-PPDA 9-1
α-PPDA 9-4

References:

Goetinck S, Waterston RH (1994) The *Caenorhabditis elegans* muscle-affecting gene *unc-87* encodes a novel thin filament-associated protein. J Cell Biol 127:79-93

Hansen, D "FEM-2 is a rapidly evolving protein phosphatase". PhD thesis. University of Alberta, 1999

Harlow E, Lane D (1988) Antibodies, a laboratory manual. Cold Spring Harbor laboratory press, Cold Spring Harbor, New York.

Hodgkin J (2002) One lucky XX male: Isolation of the first *Caenorhabditis elegans* sex-determination mutants. Genetics 162:1501-1504

Jackle-Baldwin P. "Expression studies of *fem-2* gene products". MSc thesis. University of Alberta, 1996

Stothard, PM. "Evolution of the protein phosphatase type 2C family in *Caenorhabditis*. PhD thesis. University of Alberta, 2002

Appendix B

gld-1 expression in the *C. briggsae* gonad is not sex-specific

Translation repression of germline transcripts in the hermaphrodite gonad is a common mechanism controlling two different aspects of the *C. elegans* germline development; sexual identity and meiotic progression. Not surprisingly, a role in oocyte differentiation has been found for sex-determining genes involved in the translational repression of *tra-2* (GLD-1) (Francis et al., 1995a,b) and *fem-3* (FBF-1, FBF-2, NOS-3) (Crittenden et al., 2002; Hansen et al., 2004).

Alleles of *gld-1* (GermLine Differentiation) were first isolated in a screen for XX sterility. Germ cells of *gld-1* null hermaphrodites enter meiosis but exit pachytene and return to mitotic proliferation soon after. Oocytes fail to develop and a tumor is formed in the gonad due to over proliferation. Presumably, GLD-1 activity promotes progression through meiosis by inhibiting the translation of factors important for mitotic proliferation (Francis et al., 1995). Because of the effect on controlling proliferation and inducing cellular differentiation, *gld-1* is classified as a tumor suppressor gene. Interestingly, the tumor phenotype is absent in *C. elegans gld-1* male mutants and depends on the pre-establishment of a germline commitment to the female fate (either in a wild type XX genotype or feminized X0 animals) (Francis et al., 1995b). In that respect, the roles of GLD-1 in the germline progression and oocyte maturation are ultimately controlled by the sex determination pathway.

A role for *gld-1* in sex determination was also described (see Introduction). GLD-1 promotes spermatogenesis in the L4 gonad of hermaphrodites by repressing *tra-2* mRNA translation and indirectly allowing activity of the FEM and FOG proteins (Francis et al., 1995a,b). Evidence for that is seen in *gld-1/+* animals that do not develop a tumor gonad but show a haploinsufficient effect in spermatogenesis, presumably because some TRA-2 activity remains during L4. Since the tumor phenotype depends on a previous female fate and overrides the phenotypic consequences of the sex-determining defect (feminization), null *gld-1* homozygous mutants do not show a typical Fog phenotype (Francis et al., 1995a,b).

GLD-1 is a member of the STAR-KH family of RNA-binding proteins (Clifford et al., 2000; Marin and Evans 2003; Jones and Schedl 1995) and is thought to bind over 100 different mRNA targets (Lee and Schedl 2001; Lee and Schedl 2004), including the *C. elegans* homologue of the mammalian p53 protein (Schumacher et al., 2005). In the XX gonad, mRNA targets such as the yolk receptor *rme-2*, are repressed in early meiotic cells located in the distal gonad and translated in the end of meiotic prophase in cells of the proximal gonad (Lee and Schedl 2001;Lee and Schedl 2004). The change in expression is therefore correlated with oocyte maturation. Thus, GLD-1 acts by spatially repressing the translation of specific mRNA targets. Supporting the genetic data, antibody studies indicate that *gld-1* expression in the gonad is sex-specific (Jones et al., 1996*). C. elegans* males only weakly express GLD-1 in the mitotic zone (a non-sex specific expression) whereas in hermaphrodites, GLD-1 expression is up regulated specifically in the cytoplasm of cells in the pachytene stage (*figures B1, B2*). Therefore, genetic and antibody data indicate that in *C. elegans*, *gld-1* appears to have no essential function in male germline differentiation or sex determination (Francis et al., 1995).

Germline proliferation in the *C. elegans* hermaphrodite gonad is controlled by the distal tip cell (DTC) through a Notch-like signaling pathway (Schedl 1997; Seydoux and Schedl 2001; Pepper et al., 2003a) that relies on the activation of the Notch-type receptor GLP-1 (GermLine Proliferation) by LAG-2, a DSL-type signal similar to Drosophila's Delta and Serrate proteins (Henderson et al., 1994; Crittenden et al., 1994; Tax et al., 1994; Fitzgerald and Greenwald 1995). Upon binding LAG-2, the intracellular domain of GLP-1 is cleaved and makes its way to the nucleus where it forms a transcriptional regulatory complex with LAG-1, a CSL-type transcriptional regulator (Mumm and Kopan 2000), and LAG-3/SEL-8, a protein important for the interaction of LAG-1 and the cleaved GLP-1 peptide (Seydoux and Schedl 2001; Petcherski and Kimble 2000). Aberrant GLP-1 signaling interferes with normal germline differentiation. The germline of *glp-1 null* mutants undergoes only a couple of replication rounds during the L1 larval stage before entering meiosis (Austin and Kimble 1987). Conversely, entry in meiosis is mostly inhibited in animals with constitutive activation of the GLP-1-mediated signal (Pepper et al., 2003b; Hansen et al., 2004). Correct GLP-1 signaling specifies a mitotic zone in the distal tip of the gonad responsible for repopulating the gonad with undifferentiated germ cells throughout the life of the worm (*figure B1*). Importantly, *gld-1* expression is specifically excluded from this region (*figures B1,B2*, see below)

The activities of GLD-1 and another meiosis-promoting protein, GLD-2, redundantly promote entry in meiosis by inhibiting the GLP-1-mediated proliferation (Francis et al., 1995a,b; Kadyk and Kimble 1998) in the meiotic zone. As seen above, this mechanism involves the translational repression of mitotic transcripts along the meiotic domain of the gonad. The GLD-1 effect in inhibiting proliferation is mostly prevented in the mitotic zone by the activity of the FBF proteins that bind to the *gld-1* mRNA and to repress its translation allowing mitotic division mediated by GLP-1 signaling (Crittenden et al., 2002). Like GLD-1, the FBF proteins are transcriptional regulators with a role in sex determination (see Introduction). As germ cells move away from the distal tip cell towards the loop of the gonad, the FBF control wears off and *gld-1* expression results in commitment to the meiotic fate (Jones et al., 1996).

Despite the FBF inhibition, some GLD-1 protein is present in the mitotic zone where it is thought to play a non-essential role in controlling the proliferation of premeiotic cells (Francis et al., 1995a,b). This role is not sex-specific and accounts for the detectable GLD-1 in the mitotic zone of the male gonad in *C. elegans* (Jones et al., 1996).

In contrast to other genes exclusively acting in the sex determination pathway (see Introduction), *C. elegans* and *C. briggsae* GLD-1 proteins have highly conserved sequences (Nayak et al., 2004). However, the lack of a *C. briggsae* FOG-2 orthologue and the masculinizing phenotype observed in *C. briggsae gld-1-fog-3* RNAi hermaphrodites, suggest that the sex determination role of GLD-1 has not been conserved in these two species (Nayak et al., 2004). Because FOG-2 is not necessary for the binding of GLD-1 to the *tra-2* mRNA in *C. elegans* (Clifford et al., 2000) and given the relative similarity of the 3-UTR sequences in the *tra-2* mRNA of both species (Jan et al., 1997), a scenario where *C. briggsae* GLD-1 still targets *tra-2* mRNA cannot be discarded. However, the consequence of such an interaction is not the same in these two species since instead of feminizing the worm, the knock-down *C. briggsae* GLD-1 phenotype results in sperm-producing gonads in adult hermaphrodites (Nayak et al., 2004). To account for that, either GLD-1 enhances *tra-2* translation, an unlikely role to a general translational repressor, or it does not target *tra-2* mRNA but instead binds and inhibits a male promoting factor in the *C. briggsae* germline (Nayak et al., 2004). Either way, the activities of the *C. briggsae* and *C. elegans* GLD-1 proteins have opposite outcomes in the sex determination pathways of these two species.

While characterizing the role of *gld-1* in sex determination, Nayak et al. noticed that the expression of GLD-1 in the *C. briggsae* hermaphrodite gonad is similar to the spatially restricted pattern observed in *C. elegans* (*figure B1*). Furthermore, when the sex determination pathway is set to a female mode by knocking down *fog-3*, the reduction of GLD-1 activity (*fog-3/gld-1* RNAi) causes *C. briggsae* hermaphrodites to develop the same tumor phenotype observed in *C. elegans gld-1* null mutants (Nayak et al., 2004). That indicates that contrary to the sex determination role, GLD-1 activity is important for meiotic progression in both species and dependent on a previous commitment to the female fate by the germline. Additional support for this comes from the fact that in *C. briggsae*, GLD-1 also represses translation of *rme-2* mRNA (Nayak et al., 2004). Importantly, neither the expression of GLD-1 nor the effects of *gld-1* RNAi in *C. briggsae* males have been analyzed before, probably because GLD-1 is not essential for the male germline in *C. elegans*.

Attempting to characterize the degree of sexual transformation caused by the *tra* alleles described in this work, I decided to use antibodies against molecular markers of oogenesis and spermatogenesis in immunostaining experiments (see Material and Methods). I selected a *C. elegans* antigen specific of oocyte differentiation (anti-GLD-1) and an antigen specific of spermatocyte differentiation (anti-SPE56) to track abnormal germline development due to sex determination defects. I reasoned that the sex- and tissue-specific expression patterns of these proteins would be conserved in the *C. briggsae* germline. Indeed, the *C. elegans* anti-SPE56 antibody recognizes a spermatogenesis-specific antigen in *C. briggsae* wild type hermaphrodites and males (*figures B1,B2*). Moreover, as previously described (Nayak et al., 2004), the *C. elegans* anti-GLD-1 antibody stains cells entering meiosis in the gonad of the *C. briggsae* wild type hermaphrodite (Nayak et al., 2004, this work). This expression is absent from the mitotic zone and wears off as cells move away towards the proximal gonad (*figure B1*). However, in contrast to *C. elegans*, the germline of *C. briggsae* males also expresses *gld-1* in a region populated by meiotic cells in the same stage of differentiation as those expressing *gld-1* in the hermaphrodite gonad (*figure B2*). In fact, *gld-1* expression is not sex-specific in wild type *C. briggsae* worms, opening the possibility that a role for GLD-1 also exists in the male germline.

What is the significance of *gld-1* expression in the gonad of *C. briggsae* males? RNAi or the analysis of *C. briggsae gld-1* alleles will be necessary to address this question, but interesting points can be raised concerning the extent to which the sex determination pathway controls the germline differentiation in adult *C. briggsae* worms.

In *C. elegans*, GLD-1 is a masculinizing protein acting early in the germline but its major role in meiotic progression in the adult gonad is limited to a female gonad. In comparison, *C. briggsae* GLD-1 is a feminizing protein in the L4 germline of hermaphrodites but later on controls meiosis in both female and male (presumably) gonads (*figure B3*). Given that the adoption of a tumor fate in the gonad of *C. briggsae* XX worms lacking GLD-1 activity only occurs in a previously feminized germline (*gld-1* RNAi results in a sperm-producing adult gonad but no tumor) (Nayak et al., 2004), one would not expect the GLD-1 to play a role in the male gonad. However, our immunostaining results indicate that the expression of GLD-1 is not regulated by the sex determination pathway, since the adult germline of both sexes produces GLD-1. Therefore, if as in *C. elegans*, GLD-1 activity in the *C. briggsae* gonad is important for the maturation of oocytes, but not spermatocytes, the presence of GLD-1 in the male gonad should have either a different or no functional consequences. However, such a conservation of spatially controlled expression seems to argue against a lack of function. A reasonable prediction is that GLD-1 acts to regulate spermatogenesis in *C. briggsae* males much in the same way as it controls oogenesis in XX worms (a tumor gonad would be expected in a *C. briggsae gld-1* RNAi X0 worm) even if not responding to the same developmental control. If this is true, *C. briggsae* GLD-1 would have a role in sperm production in the male (this work), but not hermaphrodite (Nayak et al., 2004). Considering that the *C. elegans* GLD-1 plays a role in spermatogenesis in the hermaphrodite, but not in the male (Francis et al., 1995a,b), this would represent an interesting evolutionary twist in the control of sexual fate and germline differentiation by GLD-1.

Reminiscent of the oocyte maturation program, meiotic progression in the *C. elegans* male germline also requires a set of translational repressors to silence mitogenic mRNA targets in cells entering meiosis (Luitjens et al., 2000). CBP-1 and FOG-1, both members of the *C. elegans* CPEB family of RNA-binding proteins, are essential for spermatogenesis but not oogenesis. The *cbp-1* expression domain in the male gonad and its predicted role during meiosis parallels that of *gld-1* in the female germline (Luitjens et al., 2000). CBP-1 protein is present in the XX gonad during L4 stage when the germline first produces sperm. After the sperm-oocyte switch, when the XX germline produces oocytes, the CBP-1 signal disappears from the gonad. Consistent with a male-specific activity, CBP-1 is also essential for correct spermatocyte differentiation in the X0 gonad since *cbp-1* males are sterile (Luitjens et al., 2000). *cbp-1* XX mutants, however, undergo normal oogenesis and can be fertilized by competent sperm. Curiously, Xenopus (CBP-3) (Stebbins-Boaz et al., 1996) and Drosophila (Orb) (Lantz et al., 1994; Chang et al., 1999) CPEB homologues are implicated in 3'-UTR regulation of mRNAs in the female germline but play no known role during spermatogenesis. Like GLD-1, Xenopus CPEB proteins target mitotic mRNAs such as cyclins and Cdk for repression to promote oocyte maturation (Stebbins-Boaz et al., 1996). In addition, the *C. elegans* homologue of the DAZ mRNA, which in humans (Reijo et al., 1996) and flies (Eberhart et al., 1996) is specifically involved in spermatogenesis, promotes oogenesis in the hermaphrodite worm while having no role on the X0 germline. In fact, it appears that either the sex-specific roles of different translational repression machinery controlling germline maturation have been reversed during the evolution of the worm or a role for these homologues exists in both sexes exists but has been missed by the analysis so far.

In the germline that has committed to a male fate (either transiently in the L4 XX germline by the activity of FOG/FEM proteins or by HER-1 in the male), CBP-1 directly binds FBF to execute the correct spermatogenesis program (Luitjens et al., 2000). As seen above, FBF itself has a specific role in promoting proliferation of stem cells in the mitotic zone of the female gonad by repressing *gld-1* translation in addition to its role in the sperm-oocyte switch when it acts with NOS-3 to repress the translation of *fem-3* mRNA. Indeed, similar to GLD-1, FBF has different effects in regulating the development and differentiation of the germline. First, it is involved in the initial sexual fate decision (feminizing role in the germline sex determination pathway) and later preventing germline differentiation by repressing meiotic entry in the adult XX gonad while promoting it in XX and X0 spermatogenesis (Luitjens et al., 2000). Thus, in *C. elegans* at least, GLD-1 and FBF are part of two antagonizing translational repression mechanisms that initially participate in setting the opposite sexual fates in the L4 germline of hermaphrodites only to promote differentiation of germlines committed to the other sex in the adult (GLD-1 in the XX gonad, FBF in the X0 gonad). The complexity of this regulation predicts that the evolution of sex-determining and germline differentiation mechanisms must be intertwined.

Regardless of the exact function of *C. briggsae* GLD-1 in the male gonad, a more complex question concerns the adoption of GLD-1 into the sex determination pathway and the consequences for the control of meiotic progression in the female and male gonads. The apparently different roles of *C. briggsae* and *C. elegans* GLD-1 in the sperm-oocyte switch (germline sex determination functions), which precedes the control of meiotic progression during development, suggests that the convergent evolution of hermaphroditism in these two species could be somehow related to the different sex-specificity of GLD-1 expression in the adult germline. However, since the role of GLD-1 in *C. briggsae* male is unknown and the differences in expression patterns of *C. elegans* and *C. briggsae* are seen in males (where no extra germline control is needed to correct sexual fate in the larva) and not hermaphrodites, the significance of this control is unclear. Until the expression pattern of GLD-1 in the gonad of other female/male nematode species is investigated (e.g. *C. remanei*), the existence of a link between the evolution of hermaphroditism and GLD-1 functions during meiotic progression in males and hermaphrodites won't be fully understood. Nevertheless, given the rapid evolution of sex determining genes in *Caenorhabditis*, the GLD-1 activity in oocyte differentiation is likely ancestral to its sex-determining role in the germline.

The investigation of genes that share roles in the sex determination and meiotic progression pathways will add to the understanding of how translational control has been applied by different fate decision mechanisms in the nematode. Recent evidence suggests that their functions do not have to be necessarily the same in both networks. For instance, besides GLD-1 and GLD-2, NOS-3 is involved in another pathway downstream of *glp-1* that also drives entry in meiosis (Hansen et al., 2004). This contrasts with the opposite roles in germline sex determination in the hermaphrodite where GLD-1 promotes male fate while NOS-3 promotes female fate (Hansen et al., 2004). The different functions of GLD-1 in sex determination and meiotic progression in *C. elegans* and *C. briggsae* further supports the notion of flexible genetic networks that evolve by adopting and discarding protein members without compromising the overall outcome of the pathway. Furthermore it opens the possibility that genetic pathways directly or indirectly dependent on the correct sexual fate are also prone to rapid divergence.

Figure B1. Immunostaining of wild type *C. elegans* (N2) (**B,C**) and *C. briggsae* (AF16) (**D,E**) hermaphrodite gonads. (**A**) Schematic diagram of the XX gonad. Germ cells differentiation in the adult XX animal progresses from the distal end ("D" in **A**) shown on the left toward the uterus region in the proximal ("P" in **A**) end shown on the right. (**B, D**) Merged DIC and anti-GLD-1 (green), anti-SPE56 (red) staining. The majority of GLD-1 staining is restricted to cells in meiotic prophase, between the mitotic stem cell population distally and oocytes finishing meiosis proximally (region between arrows, green bar in **A**). Sperm cells can be seen inside the spermatheca (arrow heads). (**C,E**) Nuclear morphology visualized with DAPI staining. DTC – distal tip cell.

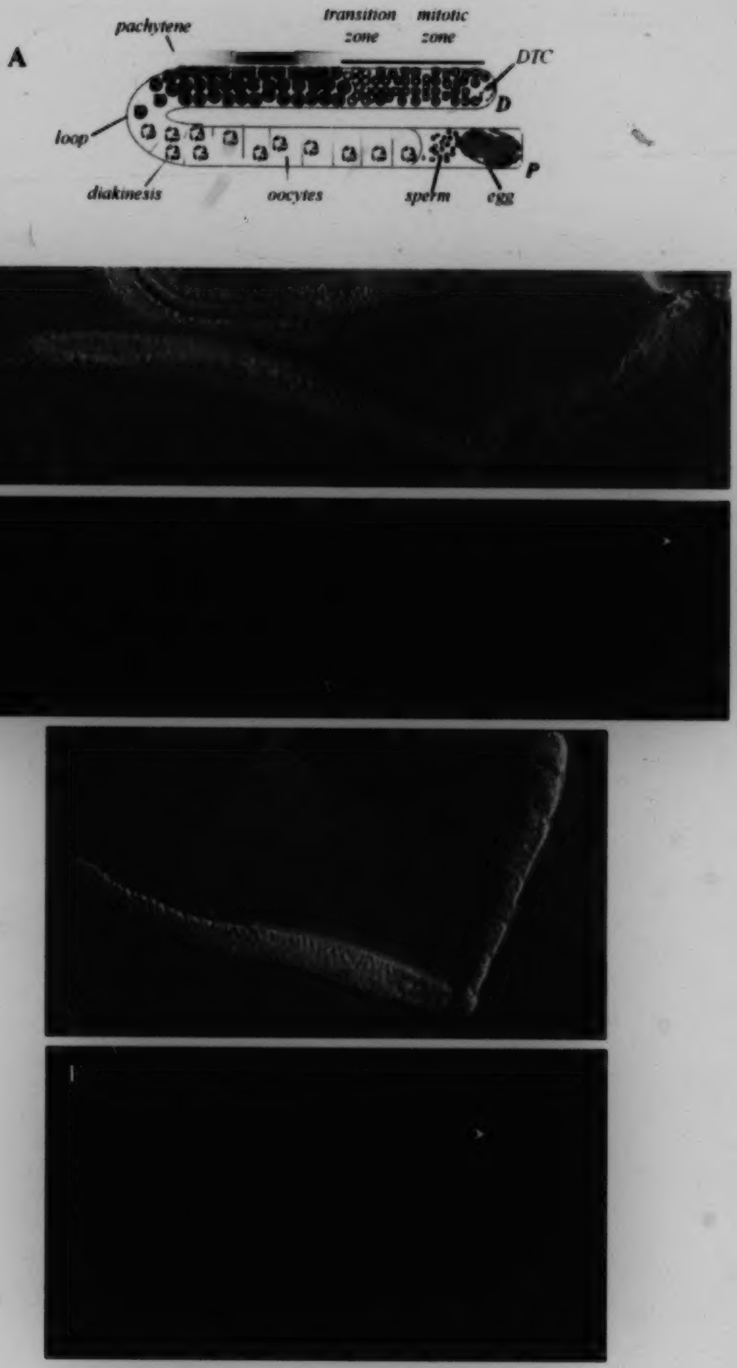
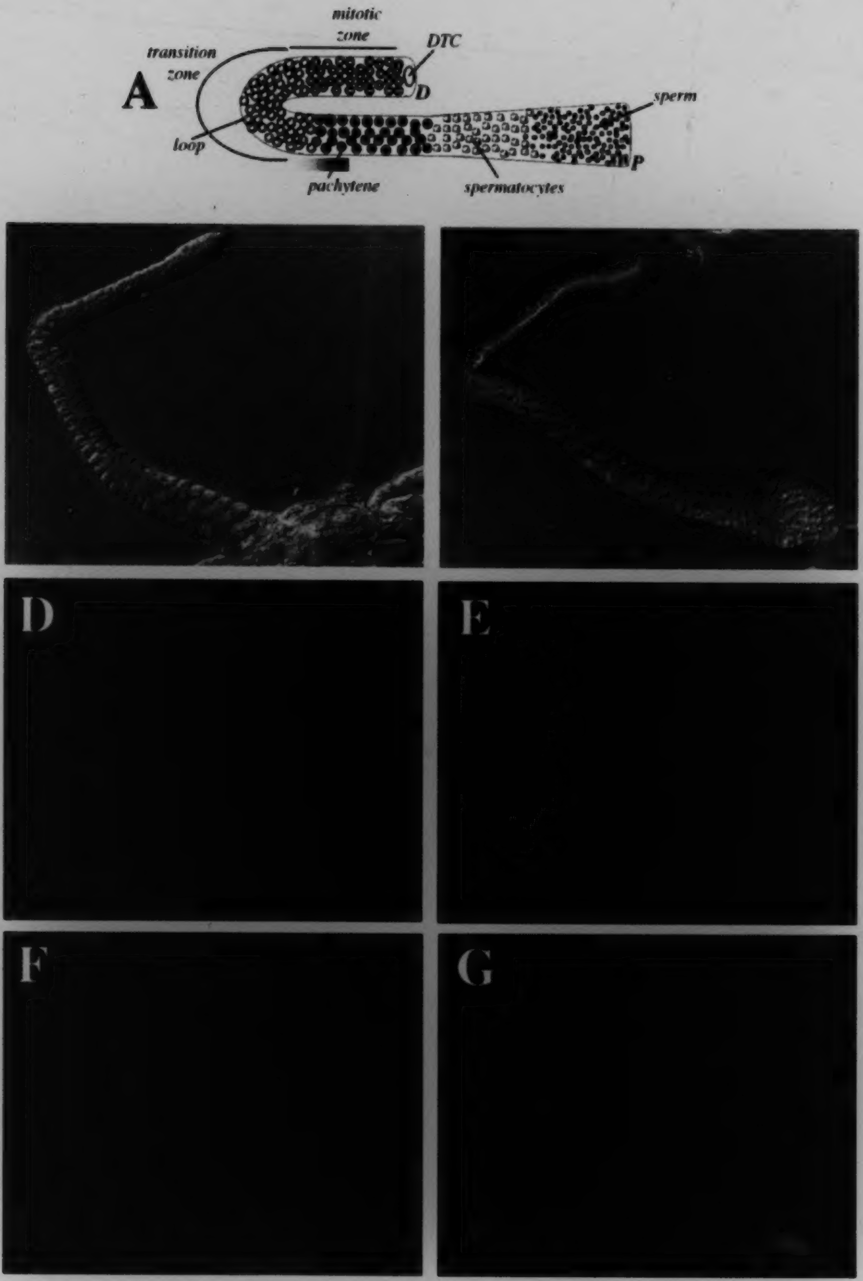

A
pachytene
transition zone
mitotic zone
DTC
D
loop
diakinesis
oocytes
sperm
egg
P

Figure B2. Immunostaining of wild type *C. elegans* (N2) (**B**,**D**,**F**) and *C. briggsae* (AF16) (**C**,**E**,**G**) male gonads. (**A**) Schematic diagram of the male gonad. Germ cells differentiation in the adult X0 animal progresses from the distal end ("D" in **A**), shown on the upper left corner, towards the posterior region ("P" in **A**) of the animal, shown on the bottom right. (**B**, **C**) Merged DIC photographs and anti-GLD-1 (green), anti-SPE56 (red) staining. Sperm in both species accumulates in the proximal gonadal region that connects to the *vas deferens* (red in **B**, **C**). In *C. elegans*, differentiating spermatocytes also express SPE56 (red in **B**). GLD-1 staining is mostly absent from meiotic cells in *C. elegans*, but present in the cells progressing through meiotic prophase in *C. briggsae*. This GLD-1+ region in the *C. briggsae* male gonad harbors cells in an equivalent state of meiosis (pachytene) as the GLD-1+ region in the hermaphrodite gonad (green bar in **A**). (**F**, **G**) Overexposed photographs confirm that the GLD-1 staining in males is specific of *C. briggsae*. (**D**,**E**) Nuclear morphology shown using DAPI. DTC- Distal tip cell.


mitotic
zone
DTC
transition
zone
A
D
sperm
loop
P
pachytene
spermatocytes
D
E
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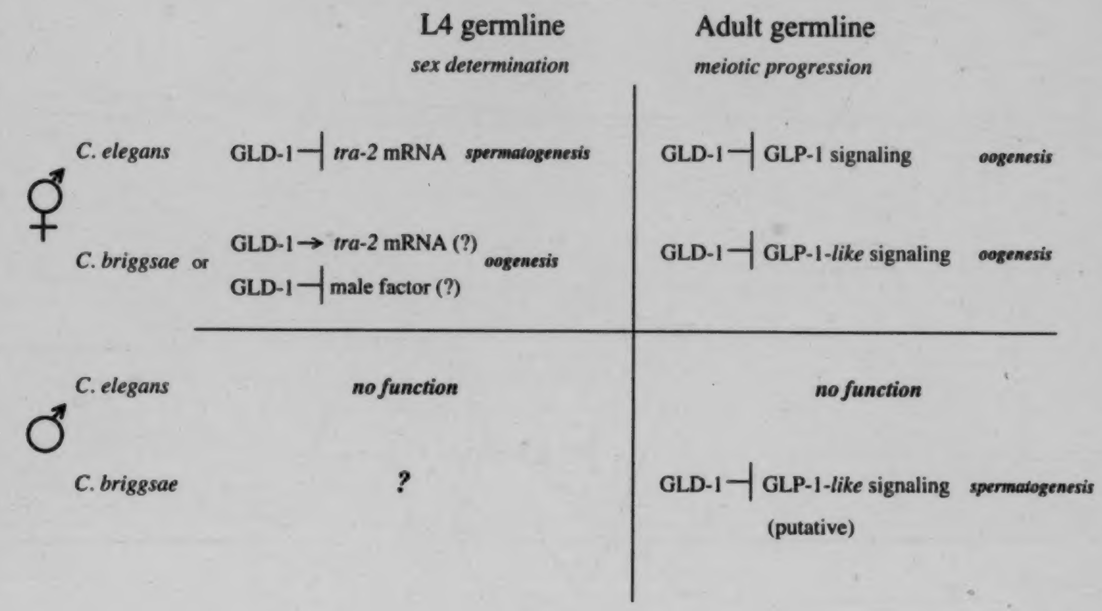
L4 germline
sex determination
Adult germline
meiotic progression
♀
C. elegans
GLD-1 ⊣ tra-2 mRNA
spermatogenesis
GLD-1 ⊣ GLP-1 signaling
oogenesis
C. briggsae
GLD-1 → tra-2 mRNA (?)
or
GLD-1 ⊣ male factor (?)
oogenesis
GLD-1 ⊣ GLP-1-like signaling
oogenesis
♂
C. elegans
no function
no function
C. briggsae
?
GLD-1 ⊣ GLP-1-like signaling
(putative)
spermatogenesis

Figure B3 – *C. elegans* and *C. briggsae* GLD-1 functions during development. In both species, GLD-1 has an early role in germline sex determination. In *C. elegans* GLD-1 activity allows a short period of spermatogenesis in the hermaphrodite by repressing *tra-2* in the germline. In *C. briggsae* GLD-1 is needed for oogenesis, either by promoting *tra-2* mRNA translation or repressing other mRNAs important for spermatogenesis. While *C. elegans* GLD-1 role in sex determination is hermaphrodite-specific, there is no data so far on the effects of GLD-1 in the *C. briggsae* male. GLD-1 also plays a role in the differentiation of the adult germline in both species. In *C. elegans*, GLD-1 promotes meiotic progression by repressing the proliferative signal from GLP-1 and this effect is restricted to the female gonad. In *C. briggsae*, GLD-1 has a role in regulating oocyte differentiation in hermaphrodites and possibly males as well.

References:

Austin J, Kimble J (1987) *glp-1* is required in the germline for regulation of the decision between mitosis and meiosis in *C. elegans*. Cell 58:565-571

Chang JS, Tan L, Schedl P (1999) The Drosophila CPEB homolog, Orb, is required for Oskar protein expression in oocytes. Dev Biol 215:91-106

Clifford R, Lee MH, Nayak S, Ohmachi M, Giorgini F, Schedl T (2000) FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the *C. elegans* hermaphrodite germline. Development 127:5265-527

Crittenden SL, Bernstein ER, Bachorik JL, Thompson BE, Gallegos M (2002) A conserved RNA-binding protein controls germline stem cell in *Caenorhabditis elegans*. Nature 417:660-663

Crittenden SL, Troemel ER, Evans TC, Kimble J (1994) GLP-1 is localized to the mitotic region of the *C. elegans* germline. Development 190:2901-2911

Eberhart CG, Maines JZ, Wasserman SA (1996) Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. Nature 381:783-785

Fitzgerald K, Greewald I (1995) Interchangeability of *Caenorhabditis elegans* DSL proteins and intrinsic signaling activity of their extracellular domains in vivo. Development 121:4275-4282

Francis R, Barton MK, Kimble J, Schedl T (1995a) *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. Genetics 139:579-606

Francis R, Maine E, Schedl T (1995b) Analysis of the multiple roles of *gld-1* in germline development: interactions with the sex determination cascade and the *glp-1* signaling pathway. Genetics 139:607-630

Hansen D, Hubbard EJ, Schedl T (2004) Multi-pathway control of the proliferation versus meiotic development decision in the *Caenorhabditis elegans* germline. Dev Biol 268:342-357

Henderson ST, Gao D, Lambie EJ, Kimble J (1994) lab-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. Development 120:2913-2924

Jan E, Yoon JW, Walterhouse D, Lannaccone P, Goodwin EB (1997) Conservation of the *C. elegans tra-2* 3'UTR translational control. EMBO J 16:6301-6313

Jones AR, Schedl T (1995) Mutations in *gld-1*, a female-specific tumor suppressor gene in *Caenorhabditis elegans* affects a conserved domain found in Src-associated protein Sam68. Genes Dev 9:1491-1504

Jones AR, Francis R, Schedl T (1996) GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. Dev Biol 180:165-183

Kadyk L, Kimble J (1998) Genetic regulation of entry into meiosis in *C. elegans*. Development 125:1803-1813

Lantz V, Chang JS, Horabin JI, Bopp D, Schedl P (1994) The Drosophila Orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes & Devel 8:598-613

Lee MH, Schedl T (2001) Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for *C. elegans* germ cell development. Genes Dev 15:2408-2420

Lee MH, Schedl T (2004) Translational repression by GLD-1 protects its mRNA targets from nonsense-mediated mRNA decay in *C. elegans*. Genes Dev 18:1047-1059

Luitjens C, Gallegos M, Kraemer B, Kimble J, Wickens M (2000) CPEB proteins control two key steps in spermatogenesis in *C. elegans*. Genes & Devel 14:2596-2609

Marin VA, Evans TC (2003) Translational repression of a *C. elegans* Notch mRNA by the STAR/KH domain protein GLD-1. Development 130:2623-2632

Mumm JS, Koplan R (2000) Notch signaling: from the outside in. Dev Biol 228:151-165

Nayak S, Goree J, Schedl T (2004) fog-2 and the evolution of self-fertile hermaphroditism in *Caenorhabditis*. Plos 3:1-15

Pepper AS, Lo TW, Killian DJ, Hall DH, Hubbard EJA (2003a) The establishment of *Caenorhabditis elegans* germline pattern is controlled by overlapping proximal and distal somatic gonad signals. Dev Biol 259:336-350

Pepper AS, Killian DJ, Hubbard EJA (2003b) Genetic analysis of *Caenorhabditis elegans glp-1* mutants suggests receptor interaction or competition. Genetics 163:115-132

Petcherski AG, Kimble J (2000) LAG-3 is a putative transcriptional activator in the *C. elegans* Notch pathway. Nature 405:364-368

Reijo R, Seligman J, Dinulos MB, Jaffe T, Brown LG, Disteche CM, Page DC (1996) Mouse autosomal homolog of DAZ, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. Genomics 35:346-352

Schedl T (1997) Developmental genetics of the germline. In *C. elegans II* (ed. D.L. Riddle, T. Blumenthal , B.J. Meyer and J.R. Priess), pp.241-269. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schumacher B, Hanazawa M, Lee MH, Nayak S, Volkmann K, Hofmann R, Hengartner M, Schedl T, Gartner A (2005) Translational repression of *C. elegans* p53 by GLD-1 regulates DNA damage-induced apoptosis. Cell 120:357-368.

Seydoux G, Schedl T (2001) The germline in *C. elegans*: origins, proliferation and silencing. Int Rev Cytol 203:139-185

Stebbins-Boaz B, Hake LE, Richter JD (1996) CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus. EMBO J 15:2582-2592

Tax FE, Yeargers JJ, Thomas JH (1994) Sequence of *C. elegans lag-2* reveals a cell signaling domain shared with *Delta* and *Serrate* of Drosophila. Nature 368:450-454